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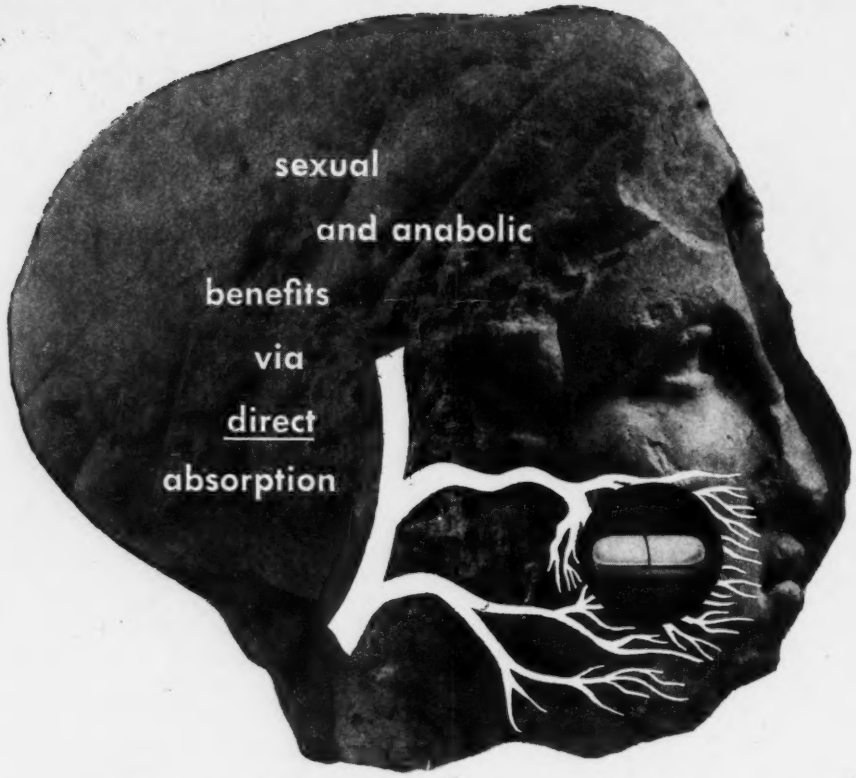

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ENDOCRINOLOGY

VOLUME 57 JULY, 1955 NUMBER 1

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THE ABILITY OF CERTAIN ADRENAL STEROIDS TO RESTORE REPRODUCTION IN ADRENALECTOMIZED FEMALE RATS

P. T. CUPPS

Department of Animal Husbandry, University of California, Davis

MANY of the early experiments concerned with the relation of the adrenal gland to reproduction were complicated because the survival time of the adrenalectomized animals was too short to allow a systematic study of reproduction. They were further complicated by the presence of accessory cortical tissue in the animals that survived. Following adrenalectomy, Martin (1932) found prolonged estrous cycles or cessation of estrus in 87.2% of 121 rats. The survival time following adrenalectomy ranged from 7 to 27 days. He reported normal cycles in 12.8% of the rats following adrenalectomy, but none of these rats survived longer than nine days. The short survival time of these latter animals was inadequate to establish normality of the cycles. Deansley (1928) reported normal reproduction in adrenalectomized rats.

Courrier, Baclesse and Marois (1953) reported a species difference with respect to the effect of cortisone on reproduction. In normal rabbits, a daily dose of 25 mg. of cortisone acetate at different stages during gestation caused either abortion or intrauterine maceration of the fetuses. In normal rats, daily injections of 12.5 mg. of cortisone did not interfere with normal gestation. Bush (1953) also reported a species difference in the balance of adrenal steroids secreted by different animals. Hisaw and Velardo (1951) found that cortisone inhibited the effect of progesterone on the decidual response in castrated pseudopregnant rats with intact adrenals. Daily injections of 1.5 mg. of cortisone reduced the diameter of the deciduamata induced by 1.5 mg. daily of progesterone by 25%, and 4.5 mg. of cortisone daily completely inhibited the response.

Folley (1952) and Cowie (1952) reported a synergistic effect of cortisone and desoxycorticosterone on lactation in adrenalectomized rats. The replacement value of the adrenal steroids on lactation was related to their

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ability to restore the food consumption of the lactating animals to normal levels. Implants of pellets which furnished 0.85 mg. of cortisone and 0.36 mg. of desoxycorticosterone per day restored lactation in the adrenalectomized rats.

EXPERIMENTAL

Virgin female rats from an inbred colony of the Long-Evans strain approximately 90 days of age were used in these experiments. They were housed in a colony where the temperature was not constant but varied between 65° and 80°F. At the beginning of the experiment, the rats were checked for normal estrous cycles. Rats with normal estrous cycles were then adrenalectomized and replacement therapy was started.

Cortisone acetate,[1] hydrocortisone acetate,[1] desoxycorticosterone acetate, and cortisone acetate combined with desoxycorticosterone acetate were used for replacement therapy. All the compounds were injected once daily subcutaneously as suspensions.

Cortisone acetate was used daily in the following concentrations: $\frac{1}{4}$ mg., $\frac{1}{2}$ mg., $1\frac{1}{4}$ mg., and $2\frac{1}{2}$ mg. Hydrocortisone acetate was used at a daily rate of $1\frac{1}{4}$ mg. and desoxycorticosterone acetate was injected at $\frac{1}{4}$, $\frac{1}{2}$, and 1 mg. daily. The combination of cortisone and desoxycorticosterone were used at $1\frac{1}{4}$ and $\frac{1}{2}$ mg. daily, respectively.

Body weights and vaginal smears were recorded daily. After the rats recovered from the surgery and showed one estrous cycle, they were bred to fertile males.

Following one gestation, all therapy was stopped. Animals that showed symptoms of insufficiency and an absence of accessory cortical tissue when autopsied were judged to be adrenalectomised. All the animals were autopsied at the end of the experiment to determine if cortical tissue was present and to count the number of implantation sites.

Five rats which had produced litters while maintained with either cortisone, hydrocortisone, or the combination of cortisone and desoxycorticosterone were checked for estrous cycles after therapy was discontinued. None of these rates cycled during the period of insufficiency. Their survival time ranged from 6 to 20 days and averaged 13 days.

The significance of the differences between the control group and each experimental group was estimated by the "t" test.

RESULTS

The ability of cortisone acetate, hydrocortisone acetate, and a combination of cortisone acetate and desoxycorticosterone acetate to restore reproduction in adrenalectomized female rats is listed in Table 1.

From the table it may be seen that the lower doses of cortisone acetate were inadequate to permit a normal number of young or number of implantations. However, cortisone acetate did allow reproduction to occur even though the females were losing weight. At the lower doses of cortisone acetate, some of the rats had difficulty at parturition. One rat of the group receiving $1\frac{1}{4}$ mg. of cortisone acetate daily died during parturition. This animal was excluded from those reported in the table because she died after the birth of one pup. The second fetus was found in the vagina and four fetuses remained in the uterus. At the highest dosage, the total number of young born alive was lower than in the control animals, but because of

[1] Cortone and Hydrocortone, courtesy of Merck & Company.

TABLE 1. THE EFFECT OF ADRENAL STEROIDS ON REPRODUCTION IN ADRENALECTOMIZED FEMALE RATS

Daily treatment	No. of rats	No. born alive	Implantation sites	Weight change during pregnancy‡ (gm.)
		(average)	(average)	(average)
Control	7	8.2	11.0	46.4
Cortisone acetate $\frac{1}{4}$ mg.	6	3.5*	5.6†	−30.8†
Cortisone acetate $\frac{1}{2}$ mg.	6	3.6*	6.2†	− 1.5†
Cortisone acetate $1\frac{1}{4}$ mg.	5	3.5	8.6*	17.2†
Cortisone acetate $2\frac{1}{2}$ mg.	5	5.8	10.0	12.8†
Hydrocortisone acetate $1\frac{1}{4}$ mg.	7	5.0	8.5*	30.7
Cortisone acetate $1\frac{1}{4}$ mg. plus Desoxycorticosterone acetate $\frac{1}{2}$ mg.	5	9.0	9.6	44.6
Desoxycorticosterone acetate $\frac{1}{4}$ mg.	4	0	0	—
Desoxycorticosterone acetate $\frac{1}{2}$ mg.	5	0	0	—
Desoxycorticosterone acetate 1 mg.	4	0	0	—
Adrenalectomized control	5	0	—	—

* Significant at .05 level.
† Significant at .01 level.
‡ Weight change of mother from day of breeding to day after parturition.

large variations this difference was not statistically significant. As the amount of cortisone acetate increased, the weight loss in the mother was less severe, but the highest dose of cortisone acetate did not allow the increase in weight shown by one-half that amount of hydrocortisone acetate.

Hydrocortisone acetate appears to be more effective in maintaining gestation than cortisone acetate, as shown by the number of young implanted and born alive. In the dose used, it seemed to even more effective in the maintenance of body weight.

Female rats maintained on desoxycorticosterone acetate did not reproduce. Their cycles were lengthened and in many cases they would not copulate when placed with males, although vaginal smears indicated that they were cycling.

When $\frac{1}{2}$ mg. of desoxycorticosterone acetate was combined with $1\frac{1}{4}$ mg. of cortisone acetate, reproductive performance appeared to be normal.

SUMMARY

In adrenalectomized female rats maintained on low doses or cortisone acetate, there was a decreased number of implantation sites, fewer young were born alive, and there was a loss of body weight during gestation.

Hydrocortisone acetate appeared to be more effective than cortisone acetate in maintaining reproduction.

Desoxycorticosterone acetate in doses varying from $\frac{1}{4}$ to 1 mg. daily does not allow normal reproduction.

A combination of cortisone acetate and desoxycorticosterone acetate is more effective in maintaining reproduction than either hormone used alone.

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GUINEA PIG COPULATORY REFLEX IN RESPONSE TO ADRENAL STEROIDS AND SIMILAR COMPOUNDS

WILLIAM W. BYRNES AND ELVA G. SHIPLEY

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan and The Endocrine Laboratories, Madison, Wisconsin

DEMPSEY, Hertz, and Young (1936) demonstrated that the copulatory reflex can be induced in ovariectomized guinea pigs by the administration of progesterone if the animals are first sensitized with estrogen. This was confirmed by Hertz, Meyer, and Spielman (1937) who also reported that pregnandione, androstenedione, dehydroandrosterone, testosterone, and estriol did not produce this effect. Van Heuverswyn, Collins, Williams, and Gardner (1939) found that desoxycorticosterone could substitute for progesterone, and Torstveit and and Mellish (1941) reported that aqueous extracts of the adrenal cortex were also effective in inducing the copulatory reflex in ovariectomized guinea pigs.

This investigation was undertaken to determine whether steroids isolated from the adrenal cortex and certain other compounds with adrenal hormone-like activity are active in producing the copulatory reflex in guinea pigs.

MATERIALS AND METHODS

Compulatory Reflex in Guinea Pigs. The method is essentially that described by Hertz, Meyer, and Spielman (1937). Ovariectomized guinea pigs were primed by administration of estrone subcutaneously for the three days preceding testing; the daily dose of estrone was 1.0 μg. contained in 0.2 cc. of 0.9% saline. On the fourth day, the test substance was administered by subcutaneous injection of the material in 0.2 cc. of corn oil. The guinea pigs were tested for estrous receptivity at five to six hours, and at eight to nine hours after treatment. The animals giving positive responses at either of the test periods are included in the results.

Progestational Activity (Corner-Allen Method). Young adult female rabbits which had been isolated for two to three weeks, and whose ovaries on examination had no corpora lutea, were used for testing progestational activity of the compounds. The rabbits were ovariectomized and treatment was begun on the same day. A biopsy specimen of the uterus was taken at operation for comparison with the autopsy specimen. Injection of the test substance in 0.2 cc. of corn oil was made once each day for five days. On the sixth day the animals were sacrificed and sections of the uterus were removed and fixed in Bouin's solution. Histological preparations of the autopsy and biopsy specimens were compared under microscopic examination for evidence of endometrial proliferation. Any specimens exhibiting proliferation were compared with preparations of uteri from rabbits treated with graded doses of crystalline progesterone.

Glycogen Deposition Activity. Steroids were assayed for liver glycogen deposition ac-

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tivity according to the method of Pabst, Sheppard, and Kuizenga (1947). This assay involves use of fasting, adrenalectomized, 150-gm. male rats injected with doses (divided into four injections) of the compound being tested or the standard preparation, crystalline hydrocortisone. The livers were analyzed for glycogen, and the ratio of potency of the unknown to the potency of the standard was calculated. By definition, hydrocortisone has 10 liver glycogen deposition units per milligram.

Adrenal-Involuting and Thymolytic Activity. Assay procedures were established for measuring and comparing the adrenal-involuting and thymolytic activity of steroids with cortisone acetate. Adrenal atrophy is considered to reflect inhibition of ACTH secretion, and thymic involution to indicate gluconeogenesis from thymic tissue. In these procedures, 21-day-old male rats of the Upjohn strain weighing about 50 gm. were injected subcutaneously daily for 6 days with 0.2 cc. of a homogenized suspension of the steroid in an aqueous diluent.[1] Cortisone acetate was given in parallel at 200 and 500 μg. per day (4 and 10 mg. per kg.). There were at least 6 rats for each dose of the steroid being tested. On the seventh day, the animals were autopsied and adrenal and thymic weights determined. When a steroid exhibited activity on this assay, the dose was adjusted so that the adrenal and thymic weights fell between the values obtained with the two dose levels of cortisone. After the proper dosage was established, the steroid was assayed at least twice and the adrenal-involuting activity and the thymolytic effects determined as a percentage of the activity of cortisone acetate.

Estrogen Assay. The estrogenic effect of steroids was measured by the response of the uteri of castrated female rats. Five immature female rats of the Upjohn strain weighing about 55 gm. were ovariectomized and injected subcutaneously daily for 7 days with a steroid suspended in an aqueous vehicle.* On the eighth day, the rats were autopsied and the uterine weights compared with those of rats injected with vehicle only, or with 17β-estradiol.

Androgen Assay. This assay tested the androgenic effect of steroids as measured by the responses of seminal vesicles and ventral prostate. Six immature male rats of the Upjohn strain weighing about 70 gm. were castrated and injected subcutaneously for 7 days with steroid suspended in an aqueous vehicle.* On the eighth day, the animals were autopsied and the seminal vesicles and prostates weighed. The weights of these organs were compared with those from rats injected with vehicle only and from rats given testosterone propionate. In this manner the androgenicity of steroids was estimated in relation to testosterone propionate.

The amorphous fraction was obtained from R. E. Knauff and E. D. Nielson, of the Endocrinology Department, and the rest of the steroids from the Chemistry Department of The Upjohn Company. 11β-hydroxyprogesterone, 11-ketoprogesterone, and 21-desoxyhydrocortisone were made from 11α-hydroxyprogesterone, a steroid which was produced by the microbiological conversion of progesterone by a process developed in our laboratories (Peterson and Murray, 1952).

RESULTS AND DISCUSSION

Table 1 lists the data obtained from testing several steroids for activity in inducing the copulatory reflex in ovariectomized, estrogen-primed guinea pigs.

Table 2 is a summary of the activity of a number of the same compounds in causing adrenal atrophy, thymic involution, and deposition of liver glycogen in the rat.

Examination of the results (Table 1) indicates that desoxycorticosterone

[1] 0.4% Tween eighty, 1.5% benzyl alcohol, 0.9% NaCl, 0.5% low viscosity carboxymethyl cellulose in distilled water.

TABLE 1. THE ACTIVITY OF STEROIDS IN CAUSING THE COPULATORY REFLEX IN ESTROGEN-PRIMED, OVARIECTOMIZED GUINEA PIGS

	Copulatory dose	Reflex assay response*
Progesterone	50 μg.	18/30
11-Ketoprogesterone	2 mg.	7/10
11β-Hydroxyprogesterone	2 mg.	7/10
11α-Hydroxyprogesterone	2 mg.	2/5
17α-Hydroxyprogesterone	2 mg.	3/9
Cortisone acetate	2 mg.	0/5
Hydrocortisone acetate	1 mg.	0/5
	2 mg.	2/5
21-Desoxyhydrocortisone	2 mg.	0/5
Corticosterone	2 mg.	3/5
11-Dehydrocorticosterone acetate	400 μg.	3/5
(Kendall's Compound A Acetate)	500 μg.	8/10
	750 μg.	4/5
	2 mg.	5/5
Desoxycorticosterone acetate	150 μg.	4/10
	200 μg.	6/10
	250 μg.	5/5
17-Hydroxy-11-desoxycorticosterone (Reichstein's Compound S)	2 mg.	1/5
4-Androstene-3,17-dione	2 mg.	0/5
Adrenosterone	2 mg.	0/5
Amorphous fraction†	150 μg.	0/5
	200 μg.	0/5

* Number of animals giving a positive response/number of animals used on test.
† Dosage expressed as equivalence of DCA on the Grollman growth-survival assay for mineralocorticoid activity.

acetate (DCA) is approximately one-fourth as active, and compound A acetate is approximately one-eighth as active, as progesterone in producing the copulatory reflex. 11-ketoprogesterone, 11β-hydroxyprogesterone, and corticosterone are about one-fortieth as active as progesterone. Compound S, hydrocortisone, 17α-hydroxyprogesterone, and 11α-hydroxyprogesterone were only slightly active at the dosage given, while cortisone, 21-desoxyhydrocortisone, 4-androstene-3,17-dione, and adrenosterone exhibited no activity at 2.0 mg. dose levels.

Both 11β-hydroxyprogesterone and 11-ketoprogesterone were lacking in progestational activity when tested on the estrous rabbit uterus at 5 mg. doses; the other steroids listed in Table 1 were not tested. From Table 2

TABLE 2. RELATIVE ACTIVITIES OF VARIOUS STEROIDS IN CAUSING ADRENAL ATROPHY, THYMIC INVOLUTION, AND LIVER GLYCOGEN DEPOSITION IN THE RAT

Steroid	Adrenal atrophy	Thymolysis	Liver glycogen deposition units
Cortisone acetate	100*	100*	5.0†
Desoxycorticosterone acetate	16.7	2.4	0.025
11β-Hydroxyprogesterone	54.0	13.7	0.36
21-Desoxyhydrocortisone	45.0	14.0	0.40
Corticosterone	41.0	39.0	3.14
11-Dehydrocorticosterone acetate	75.0	33.0	2.80
11-Ketoprogesterone	27.0	4.0	0.77
Progesterone	4.2	0.6	0.25

* Cortisone acetate activity is assigned value of 100; the activity of other steroids are percentages thereof.
† By definition, hydrocortisone has 10 liver glycogen deposition units per mg.

it can be seen that 11β-hydroxyprogesterone, 21-desoxyhydrocortisone, and 11-ketoprogesterone were active in varying degrees in causing adrenal atrophy, thymolysis, and liver glycogen deposition. These three steroids also increased glycosuria in partially depancreatized rats (Ingle, Beary, and Purmalis, 1953, a, b). None of the three compounds exhibited estrogenic activity when injected in doses as great as 100 mg. per kg. per day. When these steroids were tested for androgenic activity, 11-ketoprogesterone failed to cause prostatic or seminal vesicle stimulation in castrated male rats; 11β-hydroxyprogesterone had about 1% and 21-desoxyhydrocortisone about 3% the androgenic activity of testosterone propionate. It is of interest that although 11β-hydroxyprogesterone and 21-desoxyhydrocortisone are very similar in activity in a number of tests, only the 11β-hydroxyprogesterone was active on the copulatory reflex assay.

TABLE 3. THE ANDROGENIC ACTIVITY OF ADRENOSTERONE AND 4-ANDROSTENE-3 17-DIONE

Treatment (daily)	Body wt. (gm.)	Sem. ves. (mg.)	Vent. prost. (mg.)	Lev. ani (mg.)
Vehicle	110.5	6.2	13.8	23.1
Testosterone Propionate—100 μg.	112.0	30.8	66.9	33.8
Testosterone Propionate—125 μg.	112.0	43.4	87.9	48.6
Adrenosterone—750 μg.	107.3	30.5	70.5	39.2
4-Androstene-3,17-dione—375 μg.	108.8	15.0	72.6	33.7
4-Androstene-3,17-dione—500 μg.	105.2	30.2	140.7	26.0

Torstveit and Mellish (1931) suggested that there may be a correlation between the life-maintaining and the copulatory reflex activity of adrenal cortical extracts. This concept is not substantiated by our finding that the amorphous fraction failed to give a positive response on the copulatory reflex assay at a dose equivalent to an effective dose of desoxycorticosterone acetate based on the ratio of their activities in a growth-survival test in adrenalectomized rats.

Since the copulatory reflex is a behavioral phenomenon of a nature which might be expected to correlate with the sex hormone effects of steroids, three adrenal androgenes were assayed for their activity as androgens and in the copulatory reflex tests: 4-androstene-3,17-dione, adrenosterone, and 17α-hydroxyprogesterone.

In androgen tests, adrenosterone was found to have about 14% the activity of testosterone propionate (Table 3). The androgenic potency of 4-androstene-3,17-dione and 17α-hydroxyprogesterone does not lend itself to comparison with testosterone propionate since these steroids have preferential prostate stimulating activity (Tables 3 and 4). At a 3 mg. dose, 17α-hydroxyprogesterone caused prostatic hypertrophy, but failed to increase the seminal vesicle, levator ani, or body weight, It would appear that in this respect, 17α-hydroxyprogesterone acts in a manner similar to androsterone and androstane-3,17-dione (Hershberger, Shipley, and Meyer, 1953). The adrenal androgens, androsterone and androstenedione,

TABLE 4. THE PROSTATE STIMULATING ACTIVITY OF 17α-HYDROXYPROGESTERONE

Treatment (daily)	Body wt. (gm.)	Sem. ves. (mg.)	Vent. prost. (mg.)	Lev. ani (mg.)
Vehicle	97.8	6.5	12.1	20.9
Testosterone Propionate—125 μg.	104.5	49.4	77.1	39.5
17α-Hydroxyprogesterone—3 mg.	96.8	7.6	58.2	15.8

failed to give positive responses on the copulatory reflex assay at a dose which was forty times larger than that necessary for a response with progesterone, while 17α-hydroxyprogesterone was only slightly active. This is in accord with the findings of Hertz, Meyer, and Spielman (1937) that androgens are inactive in this assay.

SUMMARY

The copulatory reflex can be induced in estrogen-primed, ovariectomized guinea pigs by the administration of progesterone. Several adrenal steroids and similar compounds were tested and some were found to evoke this reflex. Desoxycorticosterone acetate was about one-fourth as active, and compound A acetate about one-eighth as effective as progesterone. 11-ketoprogesterone, 11β-hydroxyprogesterone, and corticosterone had about one-fortieth the activity of progesterone, while compound S, hydrocortisone, cortisone, 17α-hydroxyprogesterone, and 21-desoxyhydrocortisone were ineffective or had only slight activity. The adrenal androgens, adrenosterone and 4-androstene-3,17-dione, and the amorphous fraction did not induce this behavioral response.

The effectivenesss of adrenal steroids in inducing the copulatory reflex in ovariectomized guinea pigs is apparently not associated with gonadal hormone, glucocorticoid, mineralocorticoid, or ACTH-inhibiting activity. The data presented indicate that certain adrenal hormones may normally play a role in the copulatory reflex in guinea pigs, and by extension, may be effective in the mating behavior of other mammals.

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OBSERVATIONS ON THE CARDIOVASCULAR—RENAL EFFECTS OF 9α-CHLOROHYDROCORTISONE ACETATE IN THE RAT[1]

SYDNEY M. FRIEDMAN, CONSTANCE L. FRIEDMAN AND MIYOSHI NAKASHIMA

Department of Anatomy, The University of British Columbia, Vancouver, Canada

RECENT reports have indicated that the metabolic activity of hydrocortisone (compound F) may be considerably increased by halogen substitution in the 9α position. The original work demonstrated this with a chloro derivative (Fried and Sabo, 1953) and later studies extended the findings to the fluoro substituted compound (Fried and Sabo, 1954). A number of reports have already appeared substantiating and extending the original findings, especially those dealing with the chloro derivative (Callow *et al.*, 1954; Liddle *et al.*, 1954; Goldfien *et al.*, 1954). It now appears that 9α-chlorohydrocortisone acetate exhibits a broad spectrum of enhanced activity extending from effects on carbohydrate metabolism to marked changes in electrolyte handling.

In view of our observations concerning the blood pressure elevation which can be induced by compound F acetate in the rat (Friedman *et al.*, 1952 and 1953), it seemed of interest to subject this new derivative to our screening test for cardiovascular-renal activity.

METHODS

Male albino rats of an inbred Wistar strain were used throughout, five days after preliminary uninephrectomy. Desoxycorticosterone acetate (DCA) was administered by the subcutaneous implantation of pellets (¼ of a 75 mg. pellet), two on the first day and two 5 days later; 9α-chlorohydrocortisone acetate was injected subcutaneously as a saline suspension of microcrystals, 25 mg./cc. with added benzyl alcohol as preservative. All animals were allowed free access to tap water. Blood pressure was recorded directly from either the aorta or femoral artery through a 22 gauge needle coupled to a Sanborn electromanometer. All blood pressure determinations were carried out on animals lightly anesthetized with ether. At the conclusion of the blood pressure recording a blood sample was obtained from a side-arm in the pressure cannula for electrolyte determination and the animals immediately killed. Electrolytes were determined using a Beckman Model B flame photometer. Organs were weighed after 24 hours' fixation. Histological sections were studied after routine H and E staining.

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OBSERVATIONS

Experiment 1. The effect of 1.5 mg./day of 9α-chlorohydrocortisone acetate given subcutaneously

The selection of dose was based on our previous observation that this amount of compound F acetate given to young rats produces a well marked rise in blood pressure. The 32 uninephrectomized rats in this experiment were divided into 4 equal groups of 8 animals. Group 1 served as uninephrectomized control, Group 2 received DCA as described above, Group 3 received 1.5 mg. of 9α-chlorohydrocortisone acetate subcutaneously each day and Group 4 received both steroids. Table 1 summarizes the pertinent data.

It is obvious that 9α-chlorohydrocortisone inhibited growth completely —both when given alone and when combined with DCA. The animals given the halogenated steroid appeared so sickly that following the death of 3 animals in each of Groups 3 and 4 it was necessary to terminate the experiment on the 12th day.

Despite the sickly appearance of the rats their blood pressure (determined from the aorta) was well elevated. While DCA alone caused only a slight rise in pressure at this time, the animals receiving 9α-chlorohydrocortisone alone showed greater increments in spite of the fact that they were almost moribund. The combination of steroids showed no synergism but no firm conclusion should be drawn on this point in view of the state of the animals.

Both DCA alone and DCA combined with 9α-chlorohydrocortisone caused enlargement of the kidney relative to the size of the animal. Renal enlargement was much greater in Group 3 given the halogenated steroid alone. Histological examination of the kidneys showed that while DCA at this time had caused no obvious change, 9α-chlorohydrocortisone had produced widespread renal damage. This kidney damage was similar to that reported to follow massive dosage with DCA (Selye and Hall, 1943). It consisted of sclerotic changes in glomeruli, disruption of tubules and thickening of both the intima and media of small arterioles.

In accord with the blood pressure findings, heart weight was increased in all treated groups. This increase was least in Group 2 receiving DCA alone and greatest in Group 4 receiving both steroids.

As was to be expected, the halogenated steroid caused marked adrenal atrophy.

Electrolyte determinations were unsatisfactory because of the condition of the animals.

All in all, in view of the moderate dose level used here, this experiment indicates that this new halogenated version of compound F has marked cardiovascular-renal effects, in line with those of the parent steroid but considerably greater in degree.

TABLE 1

Group	*Experiment 1*				*Experiment 2*			
	Control	DCA	9α-chloro-hydro-cortisone acetate	DCA + 9α-chloro-hydro-cortisone acetate	Control	DCA	9α-chloro-hydro-cortisone acetate	DCA + 9α-chloro-hydro-cortisone acetate
Initial wt., grams	80 ± 2*	84 ± 1	83 ± 4	92 ± 2	63 ± 7	66 ± 2	77 ± 2	74 ± 2
Final wt., grams	132 ± 7	138 ± 5	81 ± 7	83 ± 3	172 ± 3	181 ± 8	104 ± 5	93 ± 5
	Aortic Blood Pressure mm. Hg.				Femoral Blood Pressure mm. Hg.			
Systolic	129 ± 6	145 ± 5	157 ±13	145 ± 8	128 ± 2	144 ± 5	133 ± 9	145 ±10
Diastolic	82 ± 3	88 ± 4	99 ±16	87 ±15	86 ± 4	91 ± 5	90 ± 6	104 ± 8
Mean	98 ± 4	112 ± 2	117 ±15	115 ± 9	103 ± 4	112 ± 4	103 ± 8	119 ± 9
Pulse	47	57	58	58	42	53	42	41
Kidney wt., mg./100 cm.²	272 ±13	360 ±21	441 ±27	378 ±22	282 ±13	349 ±13	387 ±28	437 ±12
Heart wt., mg./100 cm.²	187 ± 9	212 ±11	249 ±11	225 ±12	181 ± 6	199 ± 8	251 ±19	246 ± 5
Adrenal wt., mg./100 cm.²	7.6± 0.5	6.2± 0.6	2.9± 0.4	2.9± 0.4	5.9± 1.7	6.04± 0.3	3.1± 0.5	4.0± 0.6
Plasma K., mEq.					3.4± 0.06	2.4± 0.07	2.0± 0.08	2.2± 0.18
Plasma Na, mEq.					148.7± 1.2	151.8± 2.0	160.7± 5.3	156.4± 4.0

* $\epsilon = \sqrt{\frac{\Sigma x^2 - [\bar{x}(\Sigma x)]}{n(n-1)}}$

Experiment 2. The effect of 0.75 mg. of 9α-chlorohydrocortisone acetate/day given subcutaneously

In this experiment the dose of 9α-chlorohydrocortisone was halved in an effort to avoid the marked general metabolic upset observed in the first experiment. The lesser dose was still very effective in inhibiting growth but some increase in weight did occur and the rats appeared in reasonably good health. The experiment was carried for 20 days. The 32 uninephrectomized rats of this experiment were divided into 4 equal groups exactly as before, Group 1 serving as control, Group 2 receiving DCA, Group 3 receiving the daily injection of 0.75 mg. of 9α-chlorohydrocortisone and Group 4 the combined treatment with both steroids.

One important addition was made in this experiment. Following the determination of blood pressure and the withdrawal of blood for electrolyte analysis the rats were allowed three days in which to recover. A salt loading test (Friedman *et al.*, 1955) was then carried out. In brief, the method consists of administering a sodium chloride load by gavage and following the rate of excretion of sodium over measured intervals of time. Because of the introduction of this modification, direct blood pressure was recorded from the femoral artery. Table 1 presents the findings.

Again 9α-chlorohydrocortisone inhibited growth but the dose used in this experiment still permitted a small increase in weight. Growth was minimal in the group receiving this steroid together with DCA.

The plasma electrolyte study showed the usual DCA effect of a rise in sodium accompanied by a fall in potassium. The same pattern occurred with 9α-chlorohydrocortisone alone, but to a considerably greater degree. This finding agrees with the reports of others concerning the relative potency of the two steroids. The two steroids did not appear additive in their electrolyte effect in this experiment.

Since the blood pressures in this experiment were recorded from the femoral artery, they are not directly comparable with those obtained from the aorta in the first experiment. The trends are, however, quite similar. The degree of DCA induced pressure elevation is about the same in both experiments; it is present, but of a minimal order. The halogenated steroid alone this time caused no blood pressure change on the average. It should be noted, however, that 4 of the 7 surviving animals had definitely elevated pressures which are here lost in the average. Proof of this hypertensive effect even at this lower dose level is further contained in the greater blood pressure elevations observed in Group 4 receiving both steroids. It is also indicated by the marked increase in heart weight in both Groups 3 and 4.

As before, the effect of the halogenated steroid on the kidney is shown by the marked increase in renal weight. This time no histological abnormalities were noted. From our previous studies with steroids which cause both renal enlargement and damage (Friedman and Friedman, 1949), we would infer that here too the increase in kidney size represents a proportionate reduction in renal function.

The adrenal atrophy caused by 9α-chlorohydrocortisone is again seen.

The result of the salt loading test is shown graphically in Figure 1. The load consisted of 0.1 gm of NaCl in 3 ml. of distilled water. The pattern of excretion in the group receiving 9α-chlorohydrocortisone is definitely disturbed and follows the pattern which we have observed in all the forms of hypertension which we have studied, regardless of whether any electrolyte defect was apparent before loading. The peak excretion occurs rapidly so that the excess sodium is more rapidly eliminated. The total excretion, however, at the end of 3 hours is significantly less than the control, show-

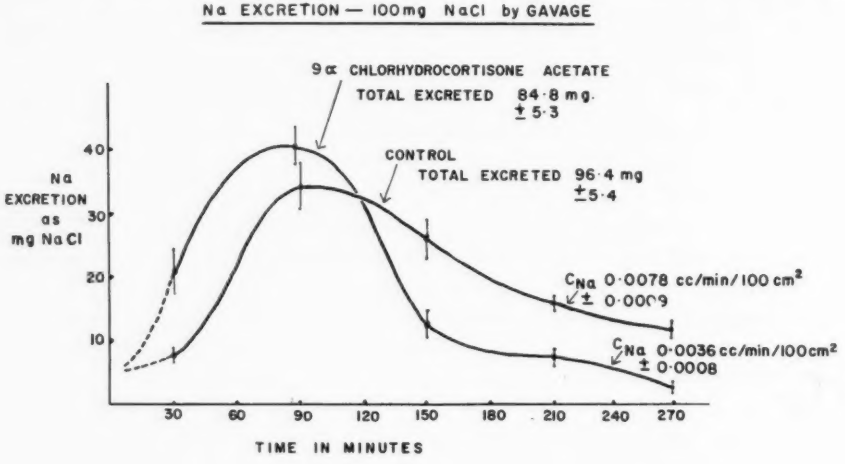

FIG. 1

ing apparent retention. The actual clearance of Na measured by referring the excretion of Na during the last hour to the plasma level of that period

$$\left(\frac{\text{urine Na}}{\text{plasma Na}}\right)$$

also shows a marked reduction in the test group, again indicating retention. Presumably, of course, the clearance of Na would be high during the peak period of Na loss. The low clearance of Na in the last period and the reduction in percentage of load excreted are consistent with the elevated plasma Na observed in the basal state. Such a retention, if it were primarily of renal origin, might be referable to a diminished filtration rate. The speed with which the excess load is excreted, however, argues against such a defect. These observations suggest strongly, but do not prove, that the electrolyte disturbance is pre-renal, with the kidney attempting to retain Na as efficiently as possible at new homeostatic levels.

DISCUSSION

The present findings confirm those of others concerning the marked increase in potency in compound F which occurs on halogenation in the 9α

position. These studies have shown that a marked effect on plasma electrolyte levels follows a relatively small dose. The change, as with DCA, is characterized by an elevation in sodium and fall in potassium. Increase in the salt load, however, is followed by a rapid excretion of the excess sodium, indicating that the animal is, with chlorohydrocortisone, functioning at a new level of electrolyte equilibrium.

The electrolyte changes are accompanied, as in the case of DCA, by an elevation in blood pressure and increase in heart weight. The involvement of the kidney in the effects of the steroid is indicated by the increase in kidney size which progresses with larger doses to frank massive damage.

From the standpoint of the general problem of the role of hormones in hypertensive processes, these findings are particularly interesting. The present report shows how a simple change in a natural product converts it to one with profound effects on electrolyte balance. The role of electrolytes in hypertension is itself a knotty problem. Effects on sodium and/or potassium metabolism do seem, however, to occupy an as yet unclear key position. Studies with the salt loading test have indicated that a disturbance in sodium metabolism exists not only in hormonal but also in renal hypertension (Friedman *et al.*, 1955). This disturbance may be minimal and not readily apparent in some instances, or obvious and marked in others. In the case of 9α-chlorohydrocortisone the implication of electrolyte metabolism is obvious and marked.

SUMMARY

The halogenated steroid, 9α-chlorohydrocortisone acetate, was administered to rats at two dose levels, 1.5 and 0.75 mg./day, for 12 and 20 days respectively.

The larger dose caused marked inhibition of growth, adrenal atrophy, hypertension with cardiac hypertrophy and renal enlargement with marked evidence of kidney damage. The experiment was terminated on the 12th day after the death of 3 out of 8 animals.

The smaller dose showed similar effects to a lesser degree. In addition, a considerable elevation of plasma sodium and depression of potassium was observed. The salt loading test showed a typical hypertensive disturbance in that the excess sodium was more rapidly eliminated although net retention occurred.

These effects of 9α-chlorohydrocortisone acetate are in the same direction and more marked than those observed with comparable doses of DCA. No particular synergism was observed, however, when the two steroids were given together.

Acknowledgment

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A NEW METHOD OF ASSAY FOR THYROTROPHIC HORMONE

D. D. ADAMS[1] AND H. D. PURVES

Endocrinology Research Department of the New Zealand Medical Research Council, Medical School, Dunedin, New Zealand

ADAMS and Purves (1953) have outlined a new method of assaying thyrotrophic hormone. The method is based upon the measurement of the action of the hormone in stimulating the secretion of thyroxine from the thyroids of guinea pigs. The measurement of the thyroxine released into the plasma is facilitated by labelling the iodine compounds of the thyroid with I^{131}. Administration of thyroxine in amounts exceeding the normal demands of the animal inhibits the secretion of thyrotrophin from the animal's own pituitary. In this paper the method is described in full, and some results obtained by use of the assay are reported.

METHOD

(*a*) *General Procedure*

Young guinea pigs, weighing 250–350 gm. are given twice-daily subcutaneous injections of 10 μg. L-thyroxine, these injections being continued until the conclusion of the assay. On the second day, each guinea pig is given 20 μc. carrier free I^{131} intraperitoneally. Following this, 72 hours are allowed to pass to let virtually all the original labelled iodide be removed from the blood and tissues by the thyroid and the kidneys. After this time the blood I^{131} level of the guinea pigs is low and steady, and the animals are ready to be injected with the thyrotrophic hormone to be assayed. The guinea pigs are warmed in front of an electric radiator, in an open mesh cage, then wrapped in a warm cloth, with only the head exposed. One of the ears is placed over the end of a perspex rod, of $\frac{1}{2}''$ diameter, bent into a right angle, with the other end collecting light from an adjacent shielded electric light bulb (Markham and Kent, 1951). This gives an excellent view of the vessels in the guinea pig's ear, the vessels being dilated owing to the warming of the animal. One of the marginal vessels is nicked with a sharp razor blade and 0.6 ml. of blood is allowed to drip into a centrifuge tube containing a drop of heparin solution. If the flow of blood stops, it can often be restarted by wiping the wound with a rough cloth. Immediately after bleeding, the animal is again held with an ear over the reflecting rod. With gentle but firm handling the guinea pig should be quietly dozing at this stage. Complete immobility is needed and a struggle with the animal is to be avoided as it often leads to a state of shock, and collapse or constriction of the ear vessels. With the guinea pig immobile and the ear stretched over the end of the reflecting rod, it is easy to inject the solution to be assayed into the marginal ear veins. The vessel running along the anterior border of the ear is particularly suitable. A sharp needle is needed and gauges 27–25 may be used. 0.5 ml. is a convenient volume to inject, but when assaying plasma

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[1] Research Fellow of the New Zealand Medical Research Council.

or serum samples it is better to inject at least 1 ml. The solution flows easily into a vessel in contrast to the marked resistance felt if the needle is outside the vessel, when an injection causes local swelling and obliteration of the vessels.

The guinea pigs are again bled from their ear veins at the time when the blood radioactivity is expected to be at its maximum following the injection of thyrotrophic hormone. This is 3 hours after intravenous injection and 5-6 hours after intraperitoneal or subcutaneous injection of the thyrotrophic hormone.

The blood samples are centrifuged to separate the plasma from the cells, and the radioactivity of the plasma is measured as described later. The counts are corrected for physical decay, and the difference between the radioactivity of the sample taken before the injection of the thyrotrophic hormone and that of the sample taken at the time of expected maximum blood radioactivity, is determined, and constitutes the response.

For convenience, one of the two daily injections of thyroxine is given following the thyrotrophin injection, the other 12 hours later.

Injection of thyrotrophic hormone and measurement of the response can be repeated on at least five successive days, in the one animal.

(*b*) *Measurement of the Plasma Radioactivity*

After centrifugation of the blood sample, 0.25 ml. of plasma is drawn up into a pipette graduated to the tip, and run out on to a 4 cm. square piece of lens paper resting on a 5 cm. square of aluminum foil. After drying under a lamp, the paper is wrapped around a thin walled beta-counter,[2] and counted. A great convenience is to have the pipette linked through a two-way tap to a filter pump, to facilitate washing it out.

Alternatively, the plasma may be drawn into the counting chamber of a flow-counter[3] and counted. These two methods are of approximately equal sensitivity, but the flow counter requires more plasma, 0.4 ml., which is a disadvantage with repeated bleedings of small animals.

A more sensitive device for counting the plasma is an end-window beta counter placed over a planchette on which a film of plasma has been dried. 0.1 ml. of plasma on a planchette gives more counts than 0.25 ml. on the lens paper, but the lens paper method is more accurate. It is difficult to ensure even spreading of the plasma on a planchette, and also, the plasma films tend to lift when they are dry, causing large errors.

The best device for counting the plasma would, no doubt, be a well type scintillation crystal. The great sensitivity of such a crystal might obviate the need for separating the plasma from the cells, and should enable the use of even smaller blood samples.

(*c*) *Scheme of Thyrotrophin Injections and Analysis of Results*

If one response only were measured from each animal used, then groups of animals could be given various dosages of thyrotrophin, a dose-response curve could be obtained and assays performed in the usual manner. As with other methods, however, the variation in responsiveness from animal to animal would necessitate the use of large numbers of animals for the achievement of a modicum of accuracy.

The ability to measure a succession of responses in each animal, enables another scheme to be employed. Table 1 shows the responses (in net counts/minute rise in radioactivity per 0.25 ml. plasma) of eight guinea pigs injected subcutaneously on four successive days with, alternately, 187.5 μg. of U.S.P. Thyrotrophin Reference Substance and 125 μg. of proposed International Standard Thyrotrophin. An analysis of the variance of this symmetrical arrangement is shown in Table 2, where it is seen that the variance between animals is much greater than that occurring from day to day with

[2] B.6. beta counter made by 20th Century Electronics, London.

[3] F10 flow counter made by 20th Century Electronics, London.

TABLE 1. THE RESPONSES, IN NET COUNTS PER MINUTE RISE IN BLOOD RADIOACTIVITY PER 0.25 ML. PLASMA, OF 8 GUINEA PIGS, TO DAILY SUBCUTANEOUS INJECTIONS OF ALTERNATELY 187.5 MICROGRAMS U.S.P. THYROTROPHIN REFERENCE SUBSTANCE AND 125 MICROGRAMS PROPOSED INTERNATIONAL STANDARD THYROTROPHIN (I.S.)

	Responses			
	Day			
	1	2	3	4
Dose in micrograms	187.5 U.S.P.	125 I.S.	187.5 U.S.P.	125 I.S.
Animal No. 21	35.2	50.9	42.7	38.0
22	47.8	139.4	110.2	141.3
23	74.5	99.6	94.6	80.6
24	59.4	126.6	118.0	128.6
Dose in micrograms	125 I.S.	187.5 U.S.P.	125 I.S.	187.5 U.S.P.
Animal No. 25	23.6	32.9	43.4	45.4
26	53.4	59.8	75.9	41.8
27	74.0	54.3	56.3	33.4
28	15.7	29.5	30.0	15.6

each animal. Analyses of variance were made of the results of three similar experiments (including two where the thyrotrophin was given intravenously) and in every instance the variance between animals was much greater than that between days. This means that if two preparations are to be compared, it is better to do so by measuring two responses in a single animal than by measuring a single response in each of two animals.

Accordingly, it was decided that responses would be compared only with other responses obtained from the same animal. Thus each animal constitutes a complete assay, and in using several animals one is doing multiple assays.

As it is not possible to construct a dose-response curve for each animal, a matching technique is used. The dosage of the preparation being assayed is adjusted until the response it elicits is approximately equal to that produced by a known amount of a standard preparation. With 250–350 gm. guinea pigs it has been found that over a range of dosage from about 100–300 μg. U.S.P. Thyrotrophin Reference Substance given subcutaneously, or 10–60 μg. U.S.P. Reference Substance given intravenously, the response appears to be directly proportional to the dosage. Within these ranges a response difference up to threefold appears to indicate a proportional difference in dosage. Differences in response greater than threefold are less dependable.

Evidence for this proportionality is shown in Table 3, where it is seen that the response ratio of the two thyrotrophin preparations changes proportionately with the dose ratio, so that, in both the subcutaneous and the intravenous experiments, the estimate of the relative potency of the two preparations is approximately the same for the three

TABLE 2. TABLE OF ANALYSIS OF VARIANCE OF THE DATA SHOWN IN TABLE 1

Source of variance	Sums of squares	Degrees of freedom	Variance	Variance ratio	P value
1. Between 187.5 U.S.P. and 125 I.S.	2,488	1	2,488	8.82	<0.01
2. Between days	3,319	3	1,106	3.92	<0.05
3. Between animals	30,693	7	4,385	15.55	<0.001
4. Residual	5,633	20	282		
5. Total	42,133	31	1,359		

different dose ratios in each of the two experiments. Table 3 shows, also, that the relative potency of thyrotrophin preparations differs according to the route of administration, a phenomenon which will be discussed later.

The best sequence of injections for thyrotrophin preparations being assayed and the best estimates of the mean responses, depend on the manner in which the responsiveness of the animal varies with each successive injection of thyrotrophin. A fall in the specific activity of the labelled iodine compounds in the thyroid will cause an apparent decrease in responsiveness, as will a blood radioactivity level which is falling rapidly owing to a big response the previous day. These effects prevent the use of dosages higher than the

TABLE 3. EVIDENCE THAT THE RESPONSE RATIO IS ARITHMETICALLY PROPORTIONAL TO THE DOSAGE RATIO, FOR A LIMITED RANGE OF RESPONSE RATIO, OVER A LIMITED RANGE OF DOSAGE

No. of animals	No. of responses	Dose of U.S.P. reference substance micrograms	Dose of International Standard thyrotrophin micrograms	Dose ratio U.S.P.:I.S.	Response ratio* I.S.:U.S.P.	Estimate of potency I.S.:U.S.P. (Dose ratio × response ratio)	Combined* estimate
		a. *Thyrotrophin injected subcutaneously*					
4	10	250	250	1.0	2.24	2.24	
8	40	187.5	125	1.5	1.23	1.84	2.00
15	59	250	125	2.0	1.02	2.04	
		b. *Thyrotrophin injected intravenously*					
7	28	25	25	1.0	1.60	1.60	
6	22	18	12	1.5	1.05	1.58	1.54
3	13	63.5	31.25	2.0	0.68	1.35	

* Geometric means.

upper limits of the ranges described earlier. Conversely, an injection of thyrotrophin may potentiate the action of a second injection of thyrotrophin, causing an increase in responsiveness.

If the responsiveness of the animal is falling off linearly during the experiment, then to compare preparations A and B, the best sequence of injections is A B A B A if five responses can be measured, and A B B A if only four responses can be measured. For three responses the best sequence is A B A. The estimate of A:B for each animal, is then given by the ratio of the arithmetic means of the responses.

If the responsiveness is not changing linearly during the experiment, then other methods of calculating would theoretically be better. To apply such methods, however, it would be necessary to know in what way the responsiveness changes during the course of the experiment.

Measurements suggest that the responsiveness decreases linearly when the thyrotrophin is given intravenously, but that there is some curvature with subcutaneously given thyrotrophin, due to a potentiation of the responses to the second and third injections. Using even numbers of animals, with pairs having reverse injection schemes, (e.g., animal No. 1—A B A B A; animal No. 2—B A B A B), would eliminate any systematic error due to deviation from a linear change of responsiveness during the experiment.

With intravenous injection of the thyrotrophin, an added complication is that occasionally there will be missing values in the responses owing to failure to perform the intravenous injection satisfactorily. It was decided that any three successive responses

TABLE 4. A COMPARISON OF U.S.P. THYROTROPHIN REFERENCE SUBSTANCE WITH PROPOSED INTERNATIONAL STANDARD THYROTROPHIN (I.S.) WHEN GIVEN INTRAVENOUSLY

(a) Responses when dose U.S.P.:dose I.S. =1:1

Dose in micrograms	Responses					Ratio of average responses I.S.:U.S.P.	Log I.S.:U.S.P.
	25 U.S.P.	25 I.S.	25 U.S.P.	25 I.S.	25 U.S.P.		
Animal No. 51	43.9	60.2	29.4	—	—	1.64	0.21
55	119.7	120.5	62.2	104.9	29.4	1.60	0.20
57	72.2	88.5	27.3	43.9	25.6	1.59	0.20
59	38.4	52.4	15.8	—	—	1.93	0.29
Dose in micrograms	25 I.S.	25 U.S.P.	25 I.S.	25 U.S.P.	25 I.S.		
Animal No. 54	116.8	63.7	—	50.8	53.2	1.48	0.17
56	60.7	30.5	49.5	—	—	1.81	0.26
58	105.4	77.1	77.3	41.9	34.7	1.22	0.09

7)1.42
log average =0.203
antilog =1.60

(b) Responses when dose U.S.P.:dose I.S. =1.5:1

Dose in micrograms	Responses					Ratio of average responses I.S.:U.S.P.	×1.5	Log I.S.:U.S.P.
	18 U.S.P.	12 I.S.	18 U.S.P.	12 I.S.	18 U.S.P.			
Animal No. 41	31.5	23.4	20.8	—	—	0.89	1.34	0.13
43	8.3	6.3	3.1	3.2	0.3	1.22	1.83	0.26
45	48.7	50.2	36.4	—	—	1.18	1.77	0.25
Dose in micrograms	12 I.S.	18 U.S.P.	12 I.S.	18 U.S.P.	12 I.S.			
Animal No. 42	22.9	12.5	14.9	—	—	1.50	2.25	0.35
44	11.1	13.5	12.0	—	—	0.86	1.29	0.11
47	17.8	30.3	16.9	11.5	16.2	0.81	1.22	0.09

6)1.19
log average =0.198
antilog =1.58

(c) Responses when dose U.S.P.:dose I.S. =2:1

Dose in micrograms	Responses					Ratio of average responses I.S.:U.S.P.	×2	Log I.S.:U.S.P.
	62.5 U.S.P.	31.25 I.S.	62.5 U.S.P.	31.25 I.S.	62.5 U.S.P.			
Animal No. 31	242.1	141.7	176.6	—	—	0.68	1.36	0.13
32	198.8	101.6	132.5	70.5	92.5	0.61	1.22	0.09
Dose in micrograms	31.25 I.S.	62.5 U.S.P.	31.25 I.S.	62.5 U.S.P.	31.25 I.S.			
Animal No. 33	50.4	67.4	51.4	48.8	29.3	0.75	1.50	0.18

3)0.40
log average =0.13
antilog =1.35

Combining (a), (b) and (c) 1.42
1.19
0.40
16)3.01
0.1881

antilog =1.54

A B A, or B A B could be used, as well as A B—B A and B A—A B where the third response was missing.

After obtaining the response ratio for each animal, and correcting it according to the dose ratio, it is necessary to combine the answers from all the animals. As the answer

A:B from each animal is a ratio, and the best estimate of the mean of ratios is the geometric mean, the ratios are averaged as logarithms, to give the final answer.

In combining the ratios from all the animals used, weighting the ratio from each animal according to the number of responses obtained from that animal would probably increase the accuracy of the final answer, but the slight improvement achieved is hardly worth the extra labor.

The assay scheme is illustrated in Table 4, which shows a comparison of the relative potencies of two thyrotrophin preparations, when given intravenously.

In measuring the thyrotrophin content of blood samples taken from people or animals, great accuracy is not usually required. A satisfactory scheme for such assays, therefore, is to find two doses of a standard thyrotrophin preparation which give responses which bracket the response to the blood. The thyrotrophin content of the blood, then, lies between the greater and the smaller doses of the standard. Alternatively, blood samples may be compared with other blood samples. Two guinea pigs are usually sufficient for doing a blood assay.

RESULTS

(a) *The Sensitivity of the Method*

This depends on the route of administration. Thyrotrophin given intravenously is about 10 times as effective as when given subcutaneously and about 5 times as effective as when given intraperitoneally. As described earlier, the optimum working range for assaying thyrotrophin given subcutaneously is about 100–300 μg. U.S.P. Thyrotrophin Reference Substance. For thyrotrophin given intravenously, the optimum working range is about 10–60 μg. U.S.P. Thyrotrophin Reference Substance. The limit of detection varies with the responsiveness of the animals used, but is of the order of 2 μg. U.S.P. Reference Substance for thyrotrophin given intravenously. This is equal to 0.0001 unit.

(b) *The Accuracy of the Method—Illustrated by Comparison of U.S.P. Reference Substance and the Proposed International Standard Thyrotrophin*

When given by the subcutaneous route, the proposed International Standard is 2.00 times as active as the U.S.P. Reference Substance, with fiducial limits of 1.79 and 2.24 for $P = 0.05$, and 1.72 and 2.33 for $P = 0.01$. Only 27 guinea pigs were needed to achieve this accuracy.

When given by the intravenous route, the proposed International Standard is 1.54 times as active as U.S.P. Reference Substance, with fiducial limits of 1.40 and 1.70 for $P = 0.05$ and 1.35 and 1.76 for $P = 0.01$. Only 16 guinea pigs were needed to achieve this accuracy. The detailed results of the assay are shown in Table 4.

(c) *The Variation in the Relative Potency of Thyrotrophin Preparations According to the Route of Administration*

It is seen above that the proposed International Standard Thyrotrophin is 2.00 times as potent as U.S.P. Thyrotrophin Reference Substance, when

they are given subcutaneously, but only 1.54 times as potent when they are given intravenously. The t value for this difference is 3.28 which, with 41 degrees of freedom, gives a P value <0.01. The discrepancy may be due to a relatively slow absorption of the U.S.P. Reference Substance when given subcutaneously.

The finding that the relative potencies of the two preparations differ according to the route of the administration has a precedent with ACTH, where the relative potencies of preparations have been found to differ as much as tenfold when given by the subcutaneous and intravenous routes (Astwood, 1954).

(d) *The Thyrotrophin Content of the Pituitary of the Normal Albino Rat*

The whole pituitary gland of the normal albino rat contains the equivalent of about 10 mg. of U.S.P. Thyrotrophin Reference Substance. As the wet weight of a rat pituitary is about 10 mg., it appears that the thyrotrophin concentration in U.S.P. Thyrotrophin Reference Substance is about equal to that in an unfractionated rat pituitary.

(e) *The Thyrotrophin Content of Various Bloods*

These have not yet been investigated thoroughly, and the values reported below, being from preliminary assays, must be considered tentative, pending further work.

The thyrotrophin present in 1 ml. of plasma from thyroidectomized rats corresponds to about 80 μg. of U.S.P. Thyrotrophin Reference Substance.

Plasma from rats being treated with methylthiouracil contains the equivalent of about 20 μg. U.S.P. Reference Substance per 1 ml.

No thyrotrophin was detected in the blood of normal humans, and none was found in the blood of two cases of thyrotoxicosis with exophthalmos. Plasma from a woman with thyrotoxicosis, who had been rendered hypothyroid by methylthiouracil, contained the equivalent of about 5 μg. U.S.P. Reference Substance per 1 ml.

(f) *Thyrotrophin Activity Present in Oxytocin*

1 ml. of a preparation of the posterior pituitary hormone, oxytocin ("Pitocin," Parke, Davis), had a thyrotrophin activity equivalent to about 5 μg. U.S.P. Thyrotrophin Reference Substance. This may represent an action of oxytocin on the anterior lobe of the pituitary, or it may be due to thyrotrophin present in the preparation as an impurity.

SUMMARY

A new method of assaying thyrotrophic hormone is described. The method depends on the labelling with I^{131} of the iodine compounds in the thyroid of a guinea pig, and the inhibition of the secretion of thyrotrophin by the animal's own pituitary by the administration of adequate amounts

of thyroxine. In animals so treated, the rise in the radioactivity of the blood following an injection of thyrotrophin, is a measure of the amount of thyrotrophin injected.

The sensitivity of the method is such that good responses are obtained with 10 micrograms of U.S.P. Thyrotrophin Reference Substance, given intravenously. The limit of detection is of the order of 2 micrograms U.S.P. Thyrotrophin Reference Substance, equivalent to 0.0001 unit.

The accuracy of the method is such that when preparations were compared by the intravenous route, using 16 animals, the standard error of the mean ratio of the activities was +5%, −4%.

The thyrotrophin content of blood samples, taken from animals and people in various conditions, is reported.

Acknowledgment

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PROMOTION OF GROWTH OF PREPUTIAL GLANDS BY STEROIDS AND THE PITUITARY GROWTH HORMONE[1]

CHARLES HUGGINS, FRANK M. PARSONS[2] AND ELWOOD V. JENSEN

The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois

IN THIS paper it will be demonstrated that the preputial glands of the hypophysectomized rat grow in response to steroids possessing the same molecular features which evoke growth in the prostate. Unlike the prostatic glands of the female rat, the preputial glands are constantly present. These giant sebaceous glands are useful indicators in the biological assay in the female rat of steroids with androgenic activity. There are important synergistic relationships between steroids and pituitary proteins in the promotion of growth so that hypophysectomy is necessary for the precise characterization of growth as induced by steroids.

The growth promoting effects of steroids upon preputial glands have not been examined systematically. Korenchevsky, Dennison and Simpson (1935) discovered that both androsterone and androstenediol caused growth of the preputial glands whilst estrone neither induced growth nor blocked the stimulatory effects of androsterone. In addition Korenshevsky (1939) found that growth of the preputial glands of ovariectomized rats occurred after the administration of the following steroids: 5-androstene-3β,17β-diol; dehydro*epi*androsterone; testosterone. In hypophysectomized rats growth of the preputial gland is induced by testosterone (Noble and Collip, 1941b) and by 4-androstene-3,17-dione (Selye and Clarke, 1943).

It has been stated that no change in the weight of the preputial glands follows the removal of the ovaries, or the adrenals, or both (Noble and Collip, 1941b). However, Korenchevsky, Dennison and Simpson (1935) had found decreased (20–40%) weight after ovariectomy and Hess, Hall,

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[2] Fellow of the American Cancer Society. Present address: Leeds University, Leeds, England.

Hall and Finerty (1952) observed hypertrophy of preputial glands after adrenalectomy.

Crude pituitary extracts cause growth of the preputial glands (Noble and Collip, 1941a) and enhance the growth promoting action of testosterone (Noble and Collip, 1941b) and 5-pregnenolone (Selye and Clarke, 1943). Jacot and Selye (1952) observed that a refined ACTH induced growth in adrenalectomized-ovariectomized rats.

METHODS

The experiments were conducted under highly standardized conditions (Huggins, Jensen and Cleveland, 1954) wherein diet, light, temperature, and humidity were controlled. A strict time schedule was followed. Albino rats of the Sprague-Dawley (Holzman) strain were received from the breeder at age 22 days and fed thereafter on the synthetic high protein (18%) ration, 4C, of Wissler, Woolridge, Steffee and Cannon (1946). Adrenalectomized rats were maintained on 0.9% sodium chloride drinking fluid. Surgical operations were performed under ether at age 24 days.

Most of the steroids[3] were recrystallized before use. Steroids were injected in 0.2 ml. of sesame oil each day from age 38 to 44 days and the dosage in this paper refers to the daily amount given; there were 5 to 14 rats in each group. The injection site was the subcutaneous tissue over the scapula, and the right and left sides were used on alternate days. Dissection was carried out at 45 days. The tissues were weighed on a torsion balance, dried to constant weight and then extracted continuously for 24 hours in ethyl ether followed by petroleum ether. The solids, moisture content and extractable lipids were then calculated. The water and solid content are expressed on a fat-free basis. Histological sections were made of the prostatic glands when these structures were present.

The criteria for completeness of hypophysectomy were these: the rats must not weigh more than 70 gm. at age 38 days or more than 73 gm. at 45 days; the spleen at necropsy must weigh less than 200 mg.

Growth hormone from bovine pituitary glands was dissolved in 0.9% sodium chloride at pH 8, freshly before injection and administered twice daily.

RESULTS

Effect of ablation of organs. The preputial glands of the intact female rat at age 24 and 45 days were considerably larger than those of intact males of the same age (Table 1). Excision of the gonads of the male and female at age 24 days was followed by a slight but statistically significant decrease (*ca.* 18–30%) in size at 45 days as compared with intact controls, age 45 days. Adrenalectomy did not cause a significant deviation from the size of the preputial gland of intact rats. The combined removal of ovaries and adrenals resulted in preputial glands of the size found when ovariec-

[3] The source of the steroids is indicated in Table 2. They were generously donated by Syntex, S. A. through the courtesy of Dr. Abraham White; Dr. F. A. Travers, Ciba Pharmaceutical Products, Inc.; Dr. Edward Henderson, Schering Corporation; and Dr. T. F. Gallagher, Sloan-Kettering Institute for Cancer Research. The growth hormone of the anterior pituitary of oxen was generously supplied by Professor C. H. Li, University of California, Berkeley, California.

tomy alone was performed. Hypophysectomy produced a profound decrease in weight of preputial glands.

The water content (Table 1) was high (*ca.* 80%) when the preputial glands were small (intact males and females at 24 days; hypophysectomized females at 45 days). By comparison the water content of the preputial glands of adult animals with intact pituitary function was lower (*ca.* 75%). Large quantities of lipids were present in the preputial glands of all groups (Table 1).

TABLE 1. WEIGHT OF PREPUTIAL GLANDS AND THEIR CONTENT OF FAT AND WATER

Status and age	No.	Fat, gm. %	Solids,* gm. %	Water,* gm. %	Range, mg.	Mean, mg.	Mg./100 body weight
			a. Females				
Intact, 24 Days	8	16.8	19.4	80.6	20.2– 30.0	24.8± 3.8	46.5±5.3 $P<0.05$
Intact, 45 Days	8	12.8	24.6	75.4	57.0–103.6	81.6±14.9	53.6±8.9 $P<0.01$
Ovariectomy,† 38 Days	6	9.9	22.9	77.1	52.4– 64.0	58.8± 4.0	43.5±4.5 $P<0.05$
Ovariectomy,† 45 Days	17	12.3	25.6	74.4	50.4– 92.8	72.0±12.3	44.0±5.7 $P<0.01$
Adrenalectomy,† 45 Days	8	10.9	24.9	75.1	54.0– 96.8	71.6±19.9	57.7±11.3 $P>0.4$
Adrenalectomy and Ovariectomy,† 45 Days	9	9.2	24.7	75.3	42.0– 75.0	52.7± 9.1	41.4±4.9 $P<0.01$
Hypophysectomy,† 45 Days	10	17.2	20.2	79.8	8.4– 11.0	9.6± 1.4	16.4±2.5 $P<0.01$
			b. Males				
Intact, 24 Days	8	12.4	19.9	80.1	8.0– 36.2	16.3±10.3	28.4±18
Intact, 45 Days	8	15.7	24.2	75.8	27.2– 76.2	49.1±19.4	29.3±10 $P<0.01$
Orchiectomy,† 45 Days	8	17.6	24.1	75.9	27.2– 46.0	33.0± 6.4	20.3±4.2 $P<0.02$

* Calculated on fat-free basis. P values calculated from Fisher's (1944) table of t. ± refers to the standard error of the means.
† All operations at age 24 days.

Effect of steroid administration. a) *C_{19} steroids.* The steroids found to produce growth of the preputial glands of hypophysectomized female rats are listed in Table 2; companion data on the effect on the size of the uterus and the incidence of grossly visible prostates are given. In each case where the prostate was visible its epithelium was tall and columnar. The smallest dose of androsterone tested, 50 μg, daily, caused a 50% increase of size of the preputial glands above the glands of uninjected controls.

Hypophysectomized males and females, age 38 days and 62–64 gm. in

TABLE 2. THE PROMOTION OF GROWTH BY C_{19} STEROIDS

Compound*	No. of rats	No. with visible prostate	Uterus mean weight, mg.	Preputial glands	
				Range, mg.	Mean, mg.
I. 4-Androsten-17β-ol-3-one[1] Testosterone	14	3	107	32.6–54.0	39.8± 8.0
II. Androstane-3α,17β-diol[2]	6	3	70	24.0–50.7	37.8±12.0
III. 4-Androstene-3α,17β-diol[1]	9	6	79	23.0–53.0	36.6±10.7
IV. 4-Androstene-3β,17β-diol[1]	9	5	85	28.0–39.4	30.8± 4.3
V. Androstane-3,17-dione[1]	11	3	20.4	21.2–36.4	28.0± 5.9
VI. 4-Androstene-3,17-dione[1]	12	6	47.9	21.8–35.4	27.3± 5.3
VII. 5-Androsten-3β-ol-17-one Dehydroepiandrosterone[3]	10	3	34.5	23.0–32.2	26.5± 4.4
VIII. 5-Androstene-3β,17β-diol[1]	12	9	61.0	16.2–42.4	24.6± 8.1
IX. Androstan-3α-ol-17-one Androsterone[4]	8	3	18.6	20.2–28.0	24.3± 3.0
X. Androstan-3β-ol-17-one *Epi*androsterone[1]	9	0	22.7	13.2–29.0	21.3± 6.4
XI. 1,4-Androstadiene-3,17-dione[1]	6	0	35.2	14.4–22.2	16.4± 2.9
XII. Androstan-17β-ol[2]	7	3	22.0	11.6–17.2	14.9± 2.2
XIII. 1,4-Androstadien-17β-ol-3-one[1]	9	2	107	11.6–15.6	13.0± 1.1
XIV. 17α-Methylandrostan-17β-ol	5	2	18.9	11.4–13.6	12.5± 1.1
Solvent	10	0	19.2	7.8–11.0	9.6± 1.4

* Compounds injected at dosage of 0.5 mg. *per diem* for 7 days.
[1] Syntex, S.A., Mexico City, Mexico.
[2] Ciba Pharmaceutical Products Inc., Summit, New Jersey.
[3] Schering Corporation, Bloomfield, New Jersey.
[4] Dr. T. F. Gallagher, Sloan-Kettering Institute for Cancer Research, New York, New York.

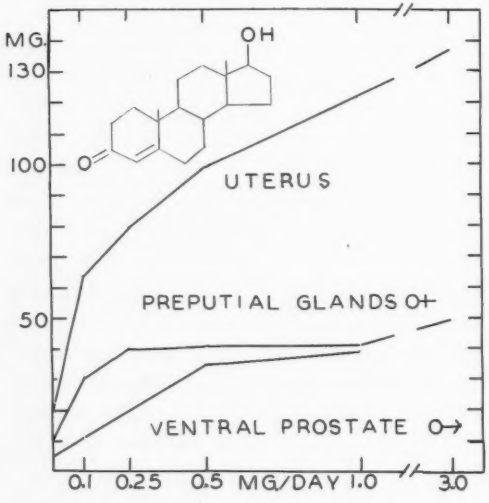

FIG. 1. Male and female hypophysectomized rats, age 38 days, were injected with testosterone (abscissae) for 7 days. The weights (at 45 days) respectively of the ventral prostate of the male and the preputial glands and uterus of the female are indicated in the abscissae. There were 5 rats in each group at each dosage level.

weight, were injected with increasing amounts of testosterone. In Figure 1 it is demonstrated that there is a close correlation of growth of the ventral prostates of the males and the preputial glands of the females.

Two steroids with *cis*-fused A:B rings were found to be devoid of growth-promoting activity; they are etiocholan-3α-ol-17-one; etiocholan-17β-ol-3-one.

b) C_{18} *steroids.* No steroids with a phenolic ring A induced growth of preputial glands in a dosage of 0.1 to 50 μg. daily. Those tested included: estrone; estradiol-3,17β; estriol; 16-*epi*estriol; 17-desoxyestradiol. More-

TABLE 3. SYNERGISM OF PITUITARY GROWTH HORMONE WITH TESTOSTERONE IN PROMOTING GROWTH OF ACCESSORY SEX GLANDS OF CASTRATE HYPOPHYSECTOMIZED MALE RATS

Status*	No. rats	Average increase body weight, gm.	Preputial glands, mg.	Seminal vesicles, mg.	Ventral prostates, mg.
I. Uninjected	8	0	10.3± 2.9 $P<0.001$	8.1±1.3 $P<0.001$	5.1±0.6 $P<0.001$
II. Growth Hormone[1]	8	+12	20.0± 1.0 $P<0.001$	8.3±1.7 $P<0.001$	5.7±1.1 $P<0.001$
III. Testosterone[2]	8	+2	27.9± 5.1 $P<0.01$	63.8±9.2 $P<0.01$	33.1±7.0 $P<0.01$
IV. Growth Hormone[1] plus testosterone[2]	8	+20	68.5±18.0	80.3±3.9	47.3±8.9

* Orchiectomy at age 22 days; hypophysectomy at age 24 days; injections from age 38 to 44 days; dissection at 45 days.

[1] Growth Hormone, 0.125 mg. twice daily.

[2] Testosterone, 0.5 mg. once daily.

± =standard deviation. *P* values were calculated, from Fisher's (1944) table of *t*, with reference to the accessory sex glands of rats in group IV injected with growth hormone plus testosterone.

over the presence of these steroids had no effect on the promotion of preputial gland growth by testosterone.

c) C_{21} *steroids.* Progesterone caused a slight increase (*ca.* 20%) of weight of preputial glands of hypophysectomized rats; the average weight of the preputial glands of 10 rats injected at 0.5 mg. dosage was 12.1±0.9 mg. Increase of progesterone to 3 mg. daily caused no additional increase in size.

The following C_{21} steroids did not produce growth of 0.5 mg. dosage; corticosterone; 11-dehydrocorticosterone; cortisone; hydrocortisone; 11-desoxy-17-hydroxycorticosterone.

Synergism of growth effect of growth hormone and testosterone. Bovine growth hormone and testosterone administered separately induced growth of the preputial glands, prostates and seminal vesicles of castrate hypophysectomized male rats (Table 3). When these hormones were injected simultaneously a considerable summation occurred in the growth of all 3 structures.

DISCUSSION

Concerning tests for masculinization of female rats Korenchevsky (1939) stated, "The easiest qualitative test is the recording of an increase in the size of the clitoris and the hypertrophy of the preputial glands. The dissection and weighing of these latter is a simple matter but unfortunately the normal variation in the weight of these glands seems to be too great for an accurate quantitative assay." This variation evidently is due to the remarkable activity of certain pituitary hormones in causing preputial gland growth and the synergism between these proteins and active steroids. These objections are circumvented by the use of hypophysectomized rats.

Of the steroids examined, only compounds in the androstane series were highly effective in promoting growth of the preputial glands. Steroids with the 5 β structure were inactive. Progesterone had definite but feeble activity.

Although the rather large variation in individual response precludes a precise evaluation of the molecular features favoring growth stimulation in the preputial gland, certain tendencies are evident from the data of Table 2. The most active steroids all possess the 17β-hydroxyl group, and, with this group present, androstane and androstene compounds with either a 3α-hydroxyl, 3β-hydroxyl or 3-ketone group are highly active. Oxidation of the 17β-hydroxyl group to a ketone results in a significant lowering of activity in certain of the more active compounds (I vs VI; II vs IX) although in other cases the 17-keto and the 17-hydroxyl compounds possess about the same potency (VII vs VIII; XI vs XIII). It is noteworthy that the steroids in which the 17β-hydroxyl compound is more active than the 17-keto analogue are those possessing a structure in ring A which favors mucification of the superficial layer of the vaginal epithelium; whereas the steroids, in which the 17-hydroxyl compound possesses the same or less activity than the corresponding keto compound, are those in which the A-ring structure favors stimulation of the basal layer of the vaginal epithelium (Huggins, Jensen and Cleveland, 1954). In all cases oxidation of the 17-hydroxyl group to a ketone group resulted in a far greater decrease in the ability of that steroid to promote uterine growth than in its effect on the preputial glands.

In the examples studied, introduction of a 1, 2 double bond into an active Δ^4-3 ketosteroid caused a marked decrease in its action on the preputial gland but not in its uterus stimulating ability (I vs XIII; VI vs XI). Monofunctional 17β-hydroxy steroids (XII; XIV) which stimulate growth of the mucus cells of the vagina (Huggins and Jensen, 1954) likewise induced growth of the preputial glands and the prostate of the female.

Promotion of growth of preputial glands by steroids could not be correlated with the induction of uterine growth but rather with the stimulation of mucification of the vaginal epithelium and with the stimulation of growth of the prostate gland.

Evidence for the latter relationship includes: 1) the high incidence of growth of the vestigial prostate of the females associated with growth of preputial glands; 2) the parallelism in growth of the prostate of the male and the preputial glands of the female when testosterone was administered; 3) of the compounds in the C_{19} series which cause growth of preputial glands those which have been tested maintain the prostatic secretion of castrate dogs[4] in the technique of Huggins and Sommer (1953). Progesterone, which has feeble activity in promoting growth of the preputial glands, has been reported to maintain the prostate of immature castrate rats (Greene, Burrill and Ivy, 1939). In sum, the preputial glands of the female are indicators of androgenic activity of steroids. Certain naturally occurring steroids, such as androsterone, in low dosage were shown to possess physiological activity by this method, whereas they appear inactive when tested on the uterus or prostate gland of the rat.

With a delicate indicator of androgenic activity available in the preputial glands of the female hypophysectomized rat, it is now possible for the first time to assay the growth promoting properties of steroids in a single group of animals. Information derived from the preputial glands usefully supplements the quantitative effects of steroids on uterus, vagina and the female prostate (when present) which are used in the assay system of Huggins, Jensen and Cleveland, 1954.

SUMMARY

Excision of the gonads of young rats causes a slight decrease in size of the preputial glands, which is unmodified by the additional removal of the adrenals. Hypophysectomy results in a profound decrease of size of preputial glands. An important synergism of growth of the preputial glands occurs when testosterone and pituitary growth hormone of bovine origin are administered together.

The preputial glands are highly responsive to steroids which induce androgenic effects in animals but not to steroids of the phenolic estrogen type. This response of the preputial glands is useful in the biological assay of steroids in the female hypophysectomized rat.

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[4] In the maintenance of prostatic secretion of the castrate dog, *epi*Androsterone (X), 50 mg. daily, is equivalent to 1 mg. of testosterone; 1,4-Androstadien-17β-ol-3-one, 25 mg., is equivalent to 2.5 mg. of testosterone; 1,4-Androstadiene-3,17-dione, 25 mg., is equivalent to 5 mg. of testosterone; Progesterone, 100 mg. daily, does not maintain the prostatic secretion in the dog.

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THE EFFECT OF ESTROGEN AND PROGESTERONE ON MAMMARY GLAND GROWTH IN THE RAT

THOMAS C. SMITH[1]

The Biological Laboratories, Harvard University, Cambridge, Mass.

EXPERIMENTS which have explored the effects of varying the amounts and ratios of estrogens and progesterone on mammary gland growth have been reported only for the rabbit (Lyons and McGinty, 1941; Scharf and Lyons, 1941) and for the mouse (Mixner and Turner, 1942a). It is surprising that these effects have not also been studied systematically on the mammary gland of the rat. The experiments reported here have attempted to assess the synergism and antagonism of estrogen and progesterone in the growth of the mammary gland of the immature ovariectomized rat.

MATERIALS AND METHODS

Female rats of the Harvard colony were fed *ad lib* a diet of Rockland pellets, and were ovariectomized at twenty-four days of age. Subcutaneous injections of estradiol-17β, or of progesterone, were begun four days later. The daily dose of estrogen was always injected in 0.05 ml. sesame oil while progesterone was always given as a solution of 20.0 mg./ml. sesame oil.

The day after the last injection, the rats were autopsied. The ventral portion of the body was shaved clean, and the skin was freed and pinned out. The abdominal-inguinal mammary glands of the right side were then removed.

The mammary tree was visualized in the manner previously described (Smith, 1954). Briefly, this consisted of spreading the glands in a Petri dish backed with a sheet of translucent weighing paper, fixing for 30 minutes in Bouin's solution and examining the partially fixed tissue with the dissecting microscope. Fixation brought out the outline of the gland so that it could be traced onto the backing paper. The area of these tracings was later measured with a compensating polar planimeter. The glands were then trimmed and weighed to the nearest 0.01 gm.

Whole gland mounts were prepared by staining with hematoxylin, processing, and then clearing in methyl salicylate. They could be alternatively mounted in gum damar as permanent slides, or stored in jars containing methyl salicylate. The stored glands were examined or photographed simply by placing them in a specially constructed chamber containing methyl salicylate. This consisted of a large ($3\times2''$) glass slide with $\frac{1}{16}$ inch thick glass strips cemented around the edge to retain the fluid. The cover, another large slide, was sealed on with silicone grease.

Samples for histological study were fixed in Zenker-formol or Zenker-acetic acid solution, processed, sectioned at eight micra and stained with hematoxylin and eosin or with Mallory's triple stain.

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[1] Present Address: Department of Pharmacology, Boston University, School of Medicine, Boston, Massachusetts.

Lobular growth was rated from the whole mounts on an arbitrary scale of one to five:

"One": Lobuli distributed very sparsely and irregularly on the ductal system, with many expanded buds and "incipient" lobuli.

"Two": Lobylar proliferation light, but more uniformly distributed.

"Three": Lobular development moderate, with some elements on every branch, and tendency of clusters to occur.

"Four": Lobular growth moderate to heavy, with many clusters and areas of packed lobuli.

"Five": Lobuli crowding together in almost all fields obscuring ductal structure and giving "packed" appearance.

The per cent of animals responding with lobulo-alveolar growth and the average degree of response for each group have been computed from these data.

Where possible, standard errors were calculated, using the formula

$$S.E. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

Tests of significance were made by the t test of "Student."

RESULTS

I. *The effects of dosage on the response of the mammary gland to estrogen alone*

Doses of 0.1, 0.5, 1.0, 2.0, 5.0 or 10.0 μg. estradiol-17β were injected daily for twelve days. Five rats were used for each dose and for the controls.

TABLE 1. EFFECT OF DAILY DOSAGE ON THE RESPONSE OF THE MAMMARY GLAND TO ESTRADIOL

Estradiol dosage (μg.)	Number of rats	Average body weight		Mammary gland			
		Increase	At autopsy	Weight (gm.)	Area (cm.²)	Lobulation	
						%	Average degree
—	5	52 ± 3.2	107 ± 5.8	0.37 ± .052	5.4 ± .17	0	0
0.1	5	46 ± 3.2	103 ± 4.6	0.52 ± .058	6.0 ± .30	0	0
0.5	5	49 ± 5.2	104 ± 4.9	0.50 ± .056	5.4 ± .41	20	0.4
1.0	5	48 ± 1.4	106 ± 3.3	0.55 ± .038	5.6 ± .27	80	1.6–2.2
2.0	5	45 ± 2.4	102 ± 3.2	0.51 ± .003	5.7 ± .35	60	1.6–2.2
5.0	5	44 ± 2.5	102 ± 3.4	0.51 ± .046	5.0 ± .50	80	2.2
10.0	5	47 ± 3.1	104 ± 5.6	0.55 ± .051	4.9 ± .35	100	3.0–3.6

In none of the groups was there any significant increase in mammary gland area. The largest dose caused a slight decrease in area ($P < 0.05$) compared to rats treated with smallest doses of estrogen (Table 1). None of the doses of estrogen caused a statistically significant increase in mammary gland weight ($P > 0.05$), although the mammary weight responses to a particular dose of estrogen were uniform, as indicated by the low standard error.

There was no alveolar development in the untreated control group or in the animals treated with 0.1 μg. estradiol daily, while the amount of alveolar growth increased moderately with doses from 0.5 to 10 μg. (Fig. 1).

Although the alveolar response within groups was quite uniform, the distribution of alveoli on the ductal structure in any single gland was uneven. This was true even with the highest dose of estrogen.

Body weight gain was depressed with all doses of estrogen employed, but was not significant ($P > 0.05$) in some cases due to group variability.

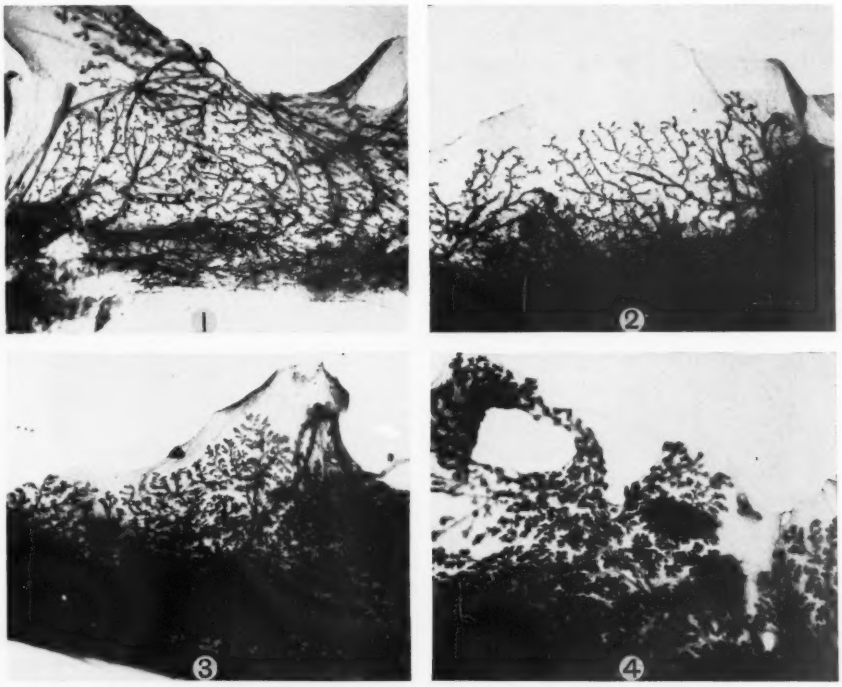

FIG. 1. The influence of dose of estradiol on mammary gland growth. Daily injections for twelve days beginning on the 28th day of age, ×4.5: 1) Ovariectomized, uninjected control. 2) 0.1 μg. Estradiol-17β control. 3) 1.0 μg. Estradiol-17β. 4) 10.0 μg. Estradiol-17β. Note the density in the center of the gland increases noticeably with dosage of estrogen.

When treated animals are compared as a group with untreated animals, the depression is highly significant ($P < 0.001$).

The primary effect of the lower doses of estradiol in this experiment was dilation of the ducts and growth and expansion of the side buds. The increase in thickness of the gland seen with the lowest dose was supplemented by further growth of buds and appearance of alveoli with increasing doses of estrogen. Since this type of growth probably occurs by displacement of fat and connective tissue, it does not necesarily result in an overall increase in volume or weight. Therefore, the histological appearance is a more meaningful index of growth than either weight or area.

II. *The effect of duration of treatment on the response of the mammary gland to estrogen*

Evaluation of mammary growth in a preliminary experiment with 36 rats suggested that the effects of estrogen on weight and area of the mammary glands appear only after two weeks or more of treatment. These observations were confirmed and extended in the experiment reported here.

Groups of four ovariectomized rats were injected for one, two, three, or four weeks with daily doses of 1.0 or 10.0 μg. estradiol-17β, while ovariec-

TABLE 2. EFFECT OF LENGTH OF TREATMENT ON THE RESPONSE OF THE MAMMARY GLAND TO ESTRADIOL

Length of treatment (days)	Number of rats	Average body weight		Mammary gland			
		Increase	At autopsy	Weight (gm.)	Area (cm.²)	Lobulation	
						%	Average degree
uninjected controls							
7	4	33 ± 0.6	91 ± 2.7	0.32 ± .025	4.6 ± .59	0	0
14	4	60 ± 6.8	119 ± 9.5	0.54 ± .070	6.7 ± .55	0	0
21	4	75 ± 2.3	136 ± 2.5	0.58 ± .058	6.4 ± .55	0	0
28	4	86 ± 4.3	141 ± 3.5	0.56 ± .047	7.1 ± .37	0	0
1.0 microgram estradiol daily							
7	4	21 ± 1.0	78 ± 3.9	0.29 ± .022	3.9 ± .26	0	0
14	4	55 ± 6.1	120 ± 5.7	0.62 ± .040	6.3 ± .71	50	0.25
21	4	60 ± 6.2	121 ± 10.2	0.71 ± .065	6.8 ± .51	50	0.5
28	4	75 ± 2.2	138 ± 4.5	1.40 ± .066	10.4 ± .49	75	1.0
10.0 micrograms estradiol daily							
7	4	21 ± 2.2	83 ± 3.8	0.30 ± .037	4.3 ± .30	75	0.5 –1.0
14	4	49 ± 5.3	110 ± 4.1	0.51 ± .016	5.8 ± .02	100	1.0 –1.75
21	4	56 ± 5.0	118 ± 4.6	0.78 ± .041	6.3 ± .28	75	1.75–2.0
28	4	60 ± 5.6	117 ± 4.4	1.00 ± .101	6.6 ± .55	100	1.75–2.25

tomized, uninjected rats of the same age were run simultaneously as controls.

The area of the glands of the control rats increased during the course of the experiment. Histological changes were confined to enlargement of the end buds and extension of the ductal tree. No lobulo-alveolar development was observed. Some increase in weight of the gland took place during the second week, but no further change was noted.

When daily treatment with 1 μg. estradiol was given, lobulo-alveolar growth appeared after two weeks, but area and weight of the glands did not become greater than those of the controls until after four weeks ($P < 0.01$) (Table 2).

Alveoli developed within a week after initiation of treatment with 10.0 μg. estradiol daily. Their development was manifestly more intense and widespread than with the lower dose of estrogen and increased with length of treatment. After three or four weeks of treatment, marked secretory

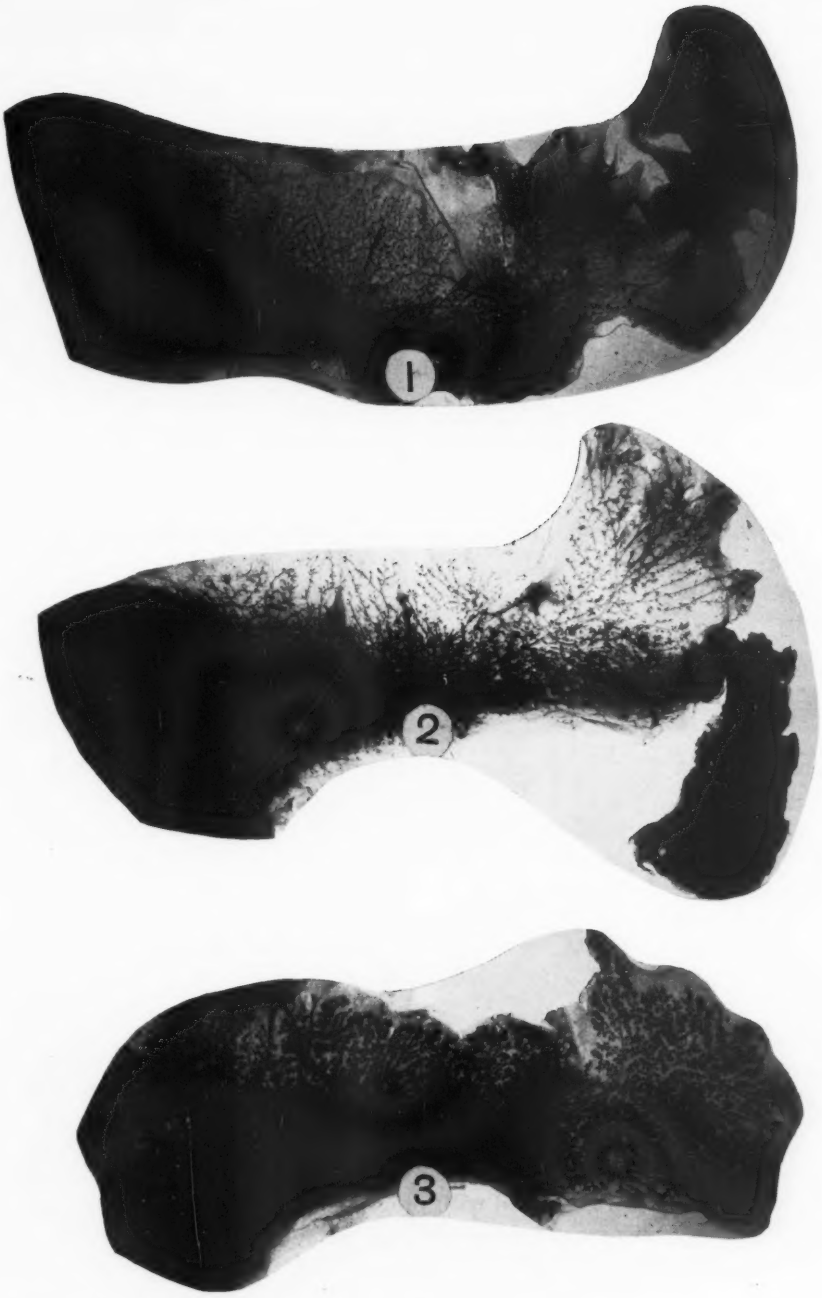

FIG. 2. Inability of estrogen priming to alter mammary gland response to progesterone alone, ×1.5: 1) No priming with estrogen; progesterone, 5.0 mg. daily for seven days from the 40th day of age. 2) Estradiol, 1.0 μg. daily for twelve days from the 28th day of age, then progesterone (5.0 mg.) daily for seven days. 3) Estradiol, 1.0 μg. daily for nineteen days from the 28th day of age, with progesterone (5.0 mg.) daily for the last seven days.

changes could also be seen. In contrast to the glands from rats treated with the lower dose of estrogen, these glands did not differ in area from the controls. Therefore, it appears that the significant ($P < 0.01$) increase in weight after four weeks treatment is due to an increase in dorso-ventral thickness or density of the organ, an observation confirmed by histological examination. No correlation between lobulo-alveolar growth and ductal extension was apparent in any of the animals on either the lower or higher dose of estrogen. Average body weight gain and the final body weight of all estrogen-treated groups were consistently less than for the controls (Table 2).

III. *Effect of priming with estrogen on the subsequent reaction to progesterone*

Rats were divided into three groups. The first was pretreated with estrogen, 1.0 μg. daily for 12 days, then injected with 5.0 mg. progesterone

TABLE 3. INFLUENCE OF COMBINATIONS OF ESTRADIOL AND PROGESTERONE ON MAMMARY GLAND GROWTH

Progesterone dosage (mg./day)	Number of rats	Average body weight		Mammary gland			
				Weight (gm.)	Area (cm.²)	Lobulation	
		Increase	At autopsy			%	Average degree
0.1 microgram estradiol daily							
1.0	3	70±3.5	113±5.5	0.47±.014	5.6±.78	0	0
2.0	3	77±5.2	124±5.0	0.48±.079	6.1±.81	67	1.0
3.0	3	51±4.4	118±1.2	0.80±.098	7.2±.59	100	2.0–3.0
4.0	3	55±1.2	120±2.5	0.82±.040	7.2±.76	100	3.6–4.0
5.0	3	60±2.1	126±4.1	0.67±.102	6.5±.27	100	3.0–4.0
1.0 microgram estradiol daily							
1.0	3	60±6.3	101±5.4	0.50±.020	6.7−.47	100	1.3
2.0	3	59±1.4	118±0.9	0.78±.034	8.3−.30	100	3.3–4.3
3.0	3	55±4.9	120±6.5	0.98±.246	7.3−.22	100	4.3–4.7
4.0	3	47±4.3	109±5.6	0.76±.092	7.0−.62	100	4.3–4.7
5.0	3	62±2.7	129±3.0	1.05±.068	8.3−.56	100	4.7
10.0 micrograms estradiol daily							
1.0	3	53±2.5	121±1.8	0.74±.144	6.3±.36	100	2.7–3.7
2.0	3	50±3.5	109±3.4	0.55±.037	6.0±.75	100	4.0
3.0	3	47±2.0	109±3.7	0.62±.030	6.5±.71	100	4.0–4.3
4.0	3	51±3.0	113±5.7	0.81±.110	6.6±.20	100	5.0
5.0	3	36±4.0	97±2.7	0.51±.060	4.6±.15	100	4.7–5.0

daily for 7 to 18 days. The second was similarly primed, then given the same doses of estrogen and progesterone concurrently for the same length of time. The third was treated with progesterone alone without any estrogen priming. Concurrent treatment with estradiol and progesterone produced grade 4 lobulo-alveolar development within seven days, and grade 5 development after twelve or eighteen days. There was slight, grade 1–2 lobulo-alveolar development in some of the estrogen-primed rats given progesterone alone for 7 days. This apparent stimulation could not be obtained consistently and was never present after 12 or 18 days. No lobular

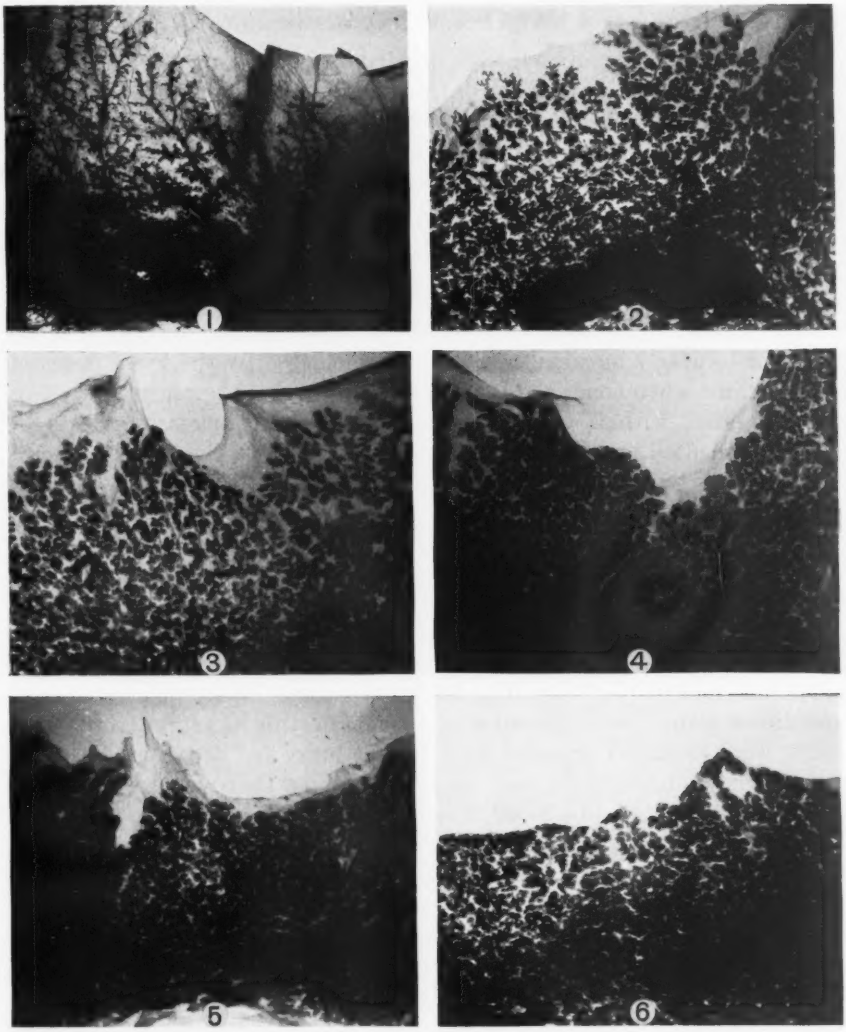

FIG. 3. Synergism of estradiol and progesterone on mammary lobulo-alveolar growth. Daily treatment for fourteen days from the 28th day of age, ×4.5: 1) Estradiol, 0.1 μg. and Progesterone, 2.0 mg. 2) Estradiol, 0.1 μg, and Progesterone, 4.0 mg. 3) Estradiol, 1.0 μg. and Progesterone, 2.0 mg. 4) Estradiol, 1.0 μg. and Progesterone, 4.0 mg. 5) Estradiol, 10 μg. and Progesterone, 2.0 mg. 6) Estradiol, 10 μg. and Progesterone, 4.0 mg.

development was seen with progesterone without estrogen priming. It appears, then, that progesterone was incapable of inducing mammary growth, either given alone or after priming with estrogen.

IV. *The interaction of estrogen and progesterone injected simultaneously*

Animals of this experiment were divided into fifteen groups of three and injected daily for 14 days. Each of three doses of estradiol (0.1, 1.0, or 10.0 μg.) was given concurrently with each of five doses of progesterone (1.0, 2.0, 3.0, 4.0, or 5.0 mm.). Rats given only 5.0 mg. progesterone per day served as controls. Except for those treated with the lowest doses of estrogen and progesterone of which only two responded, all these rats showed some lobular growth (Table 3). With 0.1 μg. of estrogen, the degree of lobular growth increased with the dose of progesterone and tended to parallel increases in weight and area of the gland. This relationship was less evident at the 1.0 μg. level and still less apparent at the 10.0 μg. level because more nearly maximal responses were elicited by the lower doses of progesterone when combined with the higher levels of estrogen.

Conversely, with a given dose of progesterone, increasing doses of estradiol resulted in greater alveolar growth, but with the highest dose of progesterone the correlation of alveolar growth with estrogen dosage is less clear-cut.

There was no case in which estrogen inhibited lobular growth, which appeared independent of any variation in area from gland to gland. The pattern of lobular growth in these glands was much more uniform than that obtained with estrogen alone. In addition, variation in response within groups was comparatively slight.

Ductal growth could be observed best in the lateral margin of the abdominal gland. Development was typical of estrogenic stimulation. The ducts were extended, covered with lateral buds and terminated by expanded end buds; but they were somewhat thinner than would be expected with estrogen stimulation alone. Progesterone alone was without effect on either ductal or alveolar growth. Nevertheless, progesterone and estrogen in the highest doses inhibited ductal growth. The stunting was apparent when the area was compared with that of any of the other groups in this experiment, or with the group treated with estrogen alone in the previous series. The data suggest, however, that progesterone usually acts to potentiate ductal growth, as reflected by an increase in mammary area and weight. Bodily weight gain and final body weight varied erratically from group to group with no specific trends.

DISCUSSION

Estrogen alone is apparently not a sufficient stimulus for complete mammary development in the rat. This fact was first noted by Turner and Schultze (1931) in their earliest experiments, and subsequently confirmed by numerous other authors (Astwood, Geschichter, and Rausch, 1937; Curtiss, 1949; Silver, 1953). In the present experiments, the response to estrogen was not "purely ductal," but involved some lobulo-alveolar growth with all but the lowest dose. Even prolonged treatment with estro-

gen did not alter the pattern of spotty distribution of alveoli on the duct system.

It is also noteworthy that progesterone alone in this dosage has been shown not to exert any significant action on mammary development in the rat (Selye, *et al.*, 1936; Curtiss, 1949; Smith and Braverman, 1953). Only one report (Selye, 1940) has appeared indicating that a much higher dose (15. mg. daily) is effective.

Although the alveoli obtained in all groups after two weeks treatment with estrogen were similar histologically, the addition of progesterone in adequate dosage increased the formation of these elements. Since progesterone alone exerted no noticeable effect on mammary growth, one can conclude that it potentiates the action of estrogen. The failure of Turner and Schultze (1931) and Astwood, *et al.* (1937) to obtain growth of the lobulo-alveolar system in the rat with similar combinations was probably due to an inadequate dose of progesterone.

Preliminary treatment with estrogen failed to alter the usual negative response to this dose of progesterone alone, while concomitant administration of both hormones produced excellent lobulo-alveolar growth. These results seem to minimize the importance of sensitization by estrogen in the progestational growth of the mammary gland. They suggest instead that both must be present during pregnancy to ensure development.

The effective dose of progesterone given with estrogen was relatively greater in the rat than that reported for either the mouse or the rabbit. Mixner and Turner (1942) injected mice with various doses of estrone and progesterone for ten days. When the *total dose* of estrone was held constant at 133 I.U., the best synergism was obtained with a total dose of 1.0–1.5 mg. progesterone. When the *total dose* of progesterone was fixed at 1.0 mg., 40–133 I.U. estrone was required for the best stimulation. Similarly, Lyons and co-workers (Lyons and McGinty, 1941; Scharf and Lyons, 1941) injected male rabbits for 28 days with variable doses of estrone and progesterone. They obtained the best synergism with a fixed daily dose of 120 I.U. estrone and 1.0 I.U. progesterone. Conversely, when the dose of progesterone was fixed at 1.0 I.U., the best synergism occurred with 240 or 960 I.U. estrone.

There is no doubt that relatively greater amount sof progesterone are required to elicit similar actions in the rat, but it is difficult to derive an optimum ratio of the two hormones from the results. Doses of 1.0 μg. estradiol and 3–5 mg. progesterone or a ratio of 1:3000–5000 may approach an optimum, or if a lesser role is assigned to the estrogen, the ratio could be 1:50,000. It is clear that fixed operative ratios, if they exist, can not be assigned from data of this type, but may ultimately be decided by analyses for the two hormones in the circulation during pregnancy.

It is interesting that a similar combination of estrogen and progesterone has also been reported the most effective for the elaboration of a "spreading

factor" in the mammary glands of this species (Elliott and Turner, 1953). Unfortunately, they did not carry out concomitant histological studies, so that direct comparison with their data is probably not valid. Furthermore, the nature of this factor, other than the fact that it did not hydrolyze hyaluronidate, has not been worked out. Failure to relate enzyme activity to histological density, DNA, or some other cellular index, leaves the important question of whether the amount of enzyme actually increases in the parenchymal tissue or merely parallels the elaboration of this tissue unanswered.

It is apparent that much smaller amounts of progesterone are necessary for the promotion or maintenance of reactions in the genital tract, such as decidual formation (Hisaw and Velardo, 1951) or the facilitation of sexual receptivity by estrogen (Boling and Blandau, 1939) than for lobulo-alveolar growth in this species. The high dose of progesterone (3–5 mg.) necessary for optimal lobulo-alveolar growth may indicate that the organ is less sensitive, or that the hormone is more rapidly metabolized in the rat than in the other species tested. In any case, the amount of progesterone needed to elicit optimal growth is not absolute, but dependent upon the amount of estrogen administered. This "sparing" action between the two hormones has been adequately demonstrated in other species and with other organ systems (Courrier, 1951).

Some mention should also be made of the role of the two hormones in ductal growth. Here the results are not too clear, and, although an increase in area and weight of the gland has been observed rather consistently when progesterone was added to estrogen treatments, the differences are small. Further work is required before progesterone can be definitely implicated in ductal growth. On the other hand, progesterone did not antagonize the profound stimulation of ductal growth by estrogen, except when both were administered in high doses.

Since estrogen did not cause a significant change in mammary gland weight and area after four weeks treatment, these indices are probably not affected by early changes, particularly by the "internal growth" of the gland. In this connection, the experiments of Reece (1950) should be mentioned. Bud growth in the mammary gland of the rat normally accompanies bodily growth. He found that male rats, put on a restricted diet to limit or eliminate these changes, were particularly sensitive to the effects of estrogen on bud growth. Nevertheless, a minimum of eight daily injections were necessary to produce detectable stimulation under these conditions. There must, therefore, be a considerable latent period between the initiation of ovarian secretions and the appearance of significant changes in the mammary gland.

The concept that mammary growth parallels body growth (or is isometric, in the terminology of relative growth analysis) but can be overridden by hormone action (or becomes positively allometric) (Cowie,

1949) is borne out by the present data. The existence of a latent period, however, suggests a gradual assumption, rather than the sharp "onset of positive allometry at the 23rd day" for the female rat proposed by Cowie (1949).

Since the recent work of Silver (1953), suggests that a dose of 1.0 μg. estradiol daily may have been more than sufficient to induce significant changes in mammary area, further experiments of this type exploring a wider range of dosages is definitely warranted.

SUMMARY

The quantitative interaction of estradiol-17β and progesterone on the mammary gland has been studied in the immature ovariectomized rat.

Estrogen alone stimulates not only ductal growth, but also a certain amount of lobulo-alveolar growth. The growth is related in amount to the dose and length of treatment, and it is characterized by a spotty distribution over the ductal tree with secretion after prolonged treatment. Only after long treatment did the area and weight of the gland show significant changes.

Progesterone alone was without effect. Nevertheless, it potentiated the action of estrogen on the lobulo-alveolar system. The amount of potentiation was directly related to the doses of both steroids. Similar potentiation of ductal growth by progesterone was suggested. In order for interaction to occur, concurrent administration is necessary. Finally, tentative optimal ratios of the two hormones have been suggested.

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ALTERATIONS OF ADRENAL CORTICAL AND OVARIAN ACTIVITY FOLLOWING HYPOTHALAMIC LESIONS

BASED ON EOSINOPHILE RESPONSE, HORMONE ASSAY AND HISTOLOGICAL EXAMINATION

GERT L. LAQUEUR, S. M. McCANN, LEON H. SCHREINER, EUGENIA ROSEMBERG, DAVID McK. RIOCH AND EVELYN ANDERSON

National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland, and the Division of Neuropsychiatry, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.

THE influence exerted by the hypothalamus on the anterior lobe of the pituitary gland has been the subject of several reviews in the last few years (Harris, 1948; Haymaker and Anderson, 1955). One of the problems of particular interest has been that of identifying hypothalamic cell aggregates or areas as centers influencing or controlling specific anterior pituitary functions. The purpose of this study was to determine whether any particular hypothalamic cell group or area was involved in the release of the adrenocorticotrophic hormone (ACTH) and the gonadotrophic hormones from the anterior lobe of the pituitary gland.

Several investigators have reported that destructive lesions in the hypothalamus in experimental animals prevent the release of ACTH which normally occurs in response to stressful situations (De Groot and Harris, 1950, 1952; Hume and Wittenstein, 1950; Porter, 1953; McCann, 1953). The methods for estimating ACTH-adrenal cortical hormone release employed by these workers were based on: 1) changes in the lymphocyte count in the rabbit (De Groot and Harris, 1950); 2) changes in the eosinophile count in the dog and cat (Hume and Wittenstein, 1950; Porter, 1953); and 3) ascorbic acid depletion of the adrenal in the rat (McCann, 1953).

According to De Groot and Harris (1950) and Porter (1953), lesions in the posterior hypothalamus abolish the ACTH responses to stress, while electrical stimulation of the same area is followed by a release of ACTH. From their data, these authors concluded that the posterior hypothalamic area is concerned in the mechanism of ACTH release from the anterior pituitary. According to Hume and Wittenstein (1950), paramedian lesions in the anterior hypothalamus and at the juncture of the middle and posterior hypothalamus in the dog abolish the adrenal cortical response to

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epinephrine and insulin and to operative trauma. Hume (1952) found, also, that lesions in the anterior portion of the median eminence (or infundibulum), the posterior portion of the tuber cinereum and the anterior portion of the mammillary bodies were effective in preventing eosinopenia following stress. McCann (1953) reported that destruction of the median eminence in the rat abolishes the eosinopenic response and also prevents the depletion of ascorbic acid from the adrenal after stress. He noted, also, that gonadal atrophy was commonly associated with median eminence lesions in the rat, confirming previous observations by Dey (1943) in the guinea pig, and Anderson, Haymaker and Rappaport (1950) in man. The influence of the hypothalamus on the gonads has been reviewed by Anderson and Haymaker (1948).

METHODS AND DESIGN OF EXPERIMENTS

Mature female cats were used. The general plan of the study was 1) to test the animal's capacity to release ACTH in response to stressful stimuli before and after a lesion had been made in the hypothalamus, 2) at the close of the experiment to collect blood from the adrenal veins and to assay the adrenal cortical hormone present, and 3) to study histologically the endocrine glands and the hypothalamus.

The fall in the eosinophile count in response to a stressful stimulus such as a laparotomy, was taken as one of the measures of ACTH-adrenal cortical hormone release. Under nembutal anesthesia, using sterile operative technique, the abdominal cavity was opened and a section of the small bowel (circa 12 inches) was withdrawn from the abdominal cavity and allowed to dry in the air for 8 minutes, and then replaced. Eosinophile counts were done immediately preceding and 4 and 6 hours after this operation. A fall in the eosinophile count 4 and 6 hours after an injection of epinephrine (1.0 mg. subcutaneous) was another measure of ACTH-adrenal cortical function. This was compared with the response in eosinophile count following an injection of ACTH (12.5 mg. intravenous).

Two epinephrine tests and a single laparotomy were carried out on each cat during the control observation period before the lesion was made in the hypothalamus. After the lesions were made, epinephrine tests were performed serially throughout the postoperative period and each animal was submitted to laparotomy once or twice. An interval of at least one week elapsed between tests.

The technique for counting eosinophiles was as follows: Ear vein blood was drawn directly into a white blood cell pipette. The diluent was that described by Spiers and Meyer (1949). For each determination duplicate blood samples were taken. A Levy counting chamber of 0.2 mm. depth was used and the cells on both sides of the chamber counted.

Under sterile operative conditions lesions were made in the hypothalamus with a Horsley-Clarke stereotaxic instrument using a unipolar electrode and a current of 3 milliamperes for 30 seconds. Bilateral paramedian lesions were made in the anterior, middle and posterior areas of the hypothalamus. If, in the postoperative period, the animals did not eat the diet offered (horsemeat, raw egg, and milk), they were fed egg and milk by stomach tube. At the termination of each experimental period, which varied between 24 and 134 days, the animal was again subjected to a stressful operation during which adrenal vein blood was collected. The procedure was as follows: The animal was anesthetized with ether and a polyethylene tube inserted into the inferior vena cava and tied below the renal veins with the tip of the tube at the level of the right adrenal.

Ligatures were placed on all the veins contributing to that part of the vena cava above and below the adrenals, except the adrenal veins. The renal arteries and veins were tied off close to the kidneys. The upper vena caval ligature was the last one tied and immediately after this the blood began to flow into the tube. Blood collections were made for exactly 15 minutes, the amount of blood collected varying from 30 to 50 ml. During the collection period, saline, with heparin added, was infused into a leg vein at the same rate as the blood flow from the vena caval pocket.

Immediately after the blood had been collected the head was perfused with saline in order to remove the intravascular blood. Then the head was perfused either with Zenker-formol or 10 per cent formalin, following which the brain and pituitary were removed and fixed in the solution with which they had been perfused. Representative pieces of all organs were fixed in a buffered formol sublimate solution and prepared for histologic examination.

Both routine and special staining procedures were used. To bring out the granulations of the pancreatic islet cells, sections were stained with aldehyde fuchsin (Gomori, 1950) and with chrome-alum-hematoxylin-phloxin (Gomori, 1941). The pituitary gland, which had been fixed in Zenker's fluid was stained by the chrome-alum-hematoxylin-phloxin method of Gomori and by the periodic acid Schiff procedure with and without a hematoxylin picric acid counterstain (Lillie, 1948).

Additional blocks of heart, kidney, liver and adrenal were fixed in 10 per cent formalin and frozen sections were stained for fat with oil red 0.

The hypothalamus was removed from the brain at autopsy and was placed in the fixing solution with which it had been perfused, but for no longer than 24 hours when Zenker-fixed. The hypothalamus was processed for paraffin embedding, using a slow dehydration schedule. Serial sections, 8 microns thick, were cut and sets of three consecutive sections were stained at regular intervals with toluidin blue, chrome-alum-hematoxylin-phloxin of Gomori (1941) and according to the periodic acid Schiff procedure of Lillie (1948). Two sections of every 50 were stained with thionin and by the Weil-Weigert method for myelin. The formalin-fixed hypothalamas was cut on a freezing microtome at 20 microns, and 2 sections of every 30 were stained with thionin and by the Weil-Weigert method for myelin. Serial drawings were made of each hypothalamus to delineate the area of hypothalamic damage.

The blood collected from the vena caval pocket was centrifuged and the plasma extracted with a mixture of 2 parts of ethyl acetate and 1 part of ethyl ether. The fat was removed by partitioning between hexane and 70% ethanol. After evaporation the residue was dissolved in a small volume of 50% propylene glycol. A bioassay of the adrenal steroids was carried out by a technique previously described (Rosemberg *et al.*, 1954).

The histological study, the eosinophile counts, and the assay of hormones were carried out in separate laboratories and the data from each were not brought together until the close of the study.

RESULTS

Effect of Hypothalamic Lesions on the Eosinophile Response to Stressful Stimuli.—The mean preoperative eosinophile count of the animals taken under resting conditions was high (1230 per cu. mm. range 175–3003). There was great variation in the eosinophile count from animal to animal but for each animal the count remained relatively constant. The handling of normal cats produced no significant change in the eosinophile count. A definite eosinopenia followed the subcutaneous injection of epinephrine dose: 1 mg.) in 115 determinations on 58 normal cats. The percentage de-

crease in eosinophile count was $31 \pm 3\%$ and $40 \pm 3\%$ four and six hours respectively after administration of the epinephrine. Laparotomy induced a very pronounced eosinopenia in the cats. This reached a maximum of $53 \pm 4\%$ decrease at six hours. The count continued to be low for 24 hours. Intravenous administration of ACTH (dose: 12.5 mg.) in 8 cats resulted in an eosinopenia with an average of $49 \pm 5\%$ decrease.

The site of the hypothalamic lesion in each cat is shown in Table 1;* the data on the altered function following hypothalamic injury are given in Table 2. Hypothalamic injury induced a significant eosinopenia in all cats,

TABLE 1. SITES OF DESTRUCTIVE LESIONS IN HYPOTHALAMUS AND NEUROHYPOPHYSIS

Cat No.	Hypothalamus								Neurohypophysis	
	Anterior			Middle			Posterior			
	Suprachiasmatic	Supraoptic	Paravent.	Med. em.	Arcuatus	Ventromed.	Mam. body	Other	Infund. stem	Infund. process
82	2+	0	0	0	0	0	0	0	0	0
75	3+	1+	1+	0	0	0	0	0	0	0
29	3+	2+	2+	0	0	0	0	0	0	0
64	3+	0	0	2+	2+	0		0	0	0
79	0	1+	3+	1+	1+	0	0	0	0	0
24	2+	2+	0	3+	3+	3+	0	0	0	0
16	2+	0	0	3+	3+	2+	0	0	3+	0
20	1+	1+	0	3+	3+	3+	0	0	3+	1+
59	0	0	0	2+	2+	0	0	0	3+	0
23	0	0	0	3+	3+	0	2+	0	0	0
42	0	0	0	3+	3+	1+	3+	0	0	3+
61	0	0	0	3+	2+	2+	2+	0	0	0
10	0	0	0	0	0	0	3+	1+	0	0
51	0	0	0	0	0	1+	3+	1+	0	0
9	0	0	0	0	0	0	2+	1+	0	0
63	0	0	0	0	0	0	3+	1+	0	0
67	0	0	0	0	0	0	2+	0	0	3+

Key: 0 = intact.
1+ = slight destruction.
2+ = at least 50% destroyed.
3+ = total destruction.

with the eosinophiles usually returning to a level near normal within a few days postoperatively. Subsequently there was no significant alteration in the eosinophile count in the group of animals as a whole; however, significant elevations or depressions of the eosinophile count seemed to occur in individual cats.

The data on the eosinophile counts which were obtained after epinephrine and after laparotomy, both before and after producing lesions in the hypothalamus are shown in Table 2. Large lesions of the posterior

* The protocols describing the lesions are published in *Research Reports*, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.

hypothalamus did not significantly alter the eosinopenic response to either epinephrine or laparotomy (Cats 10, 51, 9, 63, 76). In four of these animals, there was massive destruction of the mammillary bodies. Lesions in the suprachiasmatic area of the hypothalamus did not alter the normal eosinopenic response to epinephrine or laparotomy in cats 75 and 29. Cat 82 sickened after the lesion was placed and died before blood could be collected from adrenal vein. Destruction of the region of the median eminence and immediately adjacent hypothalamus resulted in a decrease or even abolition of epinephrine-induced eosinopenia in 23 experiments upon 9 cats.

TABLE 2. DATA ON CATS SUBJECTED TO LESIONS IN HYPOTHALAMUS AND NEUROHYPOPHYSIS

Cat No.	Survival days	Urine ml./day	Eosinophils response (% change in 6 hrs.)				Adrenal steroid output µg./min.	Data on endocrine glands		
			Epinephrine		Laparotomy			Adrenal lipids	Ovarian follicular growth	Anterior pituitary
			Before lesion	After lesion	Before lesion	After lesion				
								(1)	(2)	(3)
82	37	—	−74	− 5 (4)*	−57	−47	—	D	A	0
75	43	—	−14	−15 (3)	−12	−57	—	D	P	0
29	127	106	−29	−26 (4)	−57	− 8 (3)	1.09	D	P	0
64	73	687 (3)*	−54	− 2	−65	−53	0.00	S	A	0
79	71	491 (4)	−51	+12 (4)	−26	0	—	S	A	0
24	80	464 (4)	−44	−10 (6)	−46	− 9	—	S	A	1+
16	61	530 (1)	−48	−28 (2)	−92	− 9	0.25	S	A	0
20	61	120 (6)	−40	− 2 (4)	−76	−41	0.82	S	A	2+
59	75	685 (2)	−14	− 1 (3)	−86	−46	0.30	S	A	0
23	64	140 (5)	−46	−36	−73	−30	0.02	S	A	3+
42	24	300 (5)	−85	−53 (3)	−96	−32	0.26	S	A	2+
61	54	334 (4)	−43	−13 (4)	−49	−12 (2)	0.92	S	A	1+
10	134	134	−37	−27 (3)	−86	−40	1.42	D	P	0
51	35	205	−30 (4)	−27 (3)	−20	−44	1.39	D	P	0
9	116	—	−54 (3)	−45 (3)	−44	−46	0.85	D	P	0
63	85	135 (5)	−78	−55 (5)	−98	−93	—	D	P	0
76	67	379	−30	−29 (3)	−22	−38	—	D	P	0
83	Control						0.13	S	P	0
84	Control						1.92	D	P	0
65	Control						1.05	D	P	0
66	Control						1.32	Partial D	P	0

* Number italicized in parentheses denotes number of determinations used to compute value for urine volume and for eosinophile change, otherwise the value represents 2 determinations for epinephrine test and one for laparotomy response.
(1) Lipid depletion = D; lipid stored = S.
(2) Ovarian follicular growth arrested = A; present = P.
(3) 0 = intact; 1+ = slight destruction; 2+ at least 50% destroyed; 3+ = total destruction.

There was no recovery of the response in the two to five month survival period. The eosinopenic response to laparotomy was practically abolished in 3 of these cats. On the whole, the results indicate that the eosinopenic response to laparotomy is retained in an attenuated form after the hypothalamic injury. Laparotomy was repeated in cat 61 which showed an impaired response.

Effect of Hypothalamic Lesions on the Release of Adrenal Hormone, Lipid Storage in the Adrenal Cortex, and on the Growth of Follicles of the Ovary.— Evidence of ACTH stimulation of the adrenal cortex was based on the finding of an increase in the adrenal cortical hormone titer in adrenal venous blood together with a decrease in the stainable lipid in the adrenal cortex. The hormone titer is expressed as micrograms of cortisone per minute of blood flow.

Analysis of the data in Table 2 shows that the cats with lesions in the

suprachiasmatic area (Cat 29) and posterior area (Cats 10, 51, and 9) of the hypothalamus responded to a stressful stimulus with an outpouring of adrenal steroids varying from 0.85 to 1.42 μg. per minute. Correlated with this high titer of adrenal steroids in the blood was a depletion of the stain-

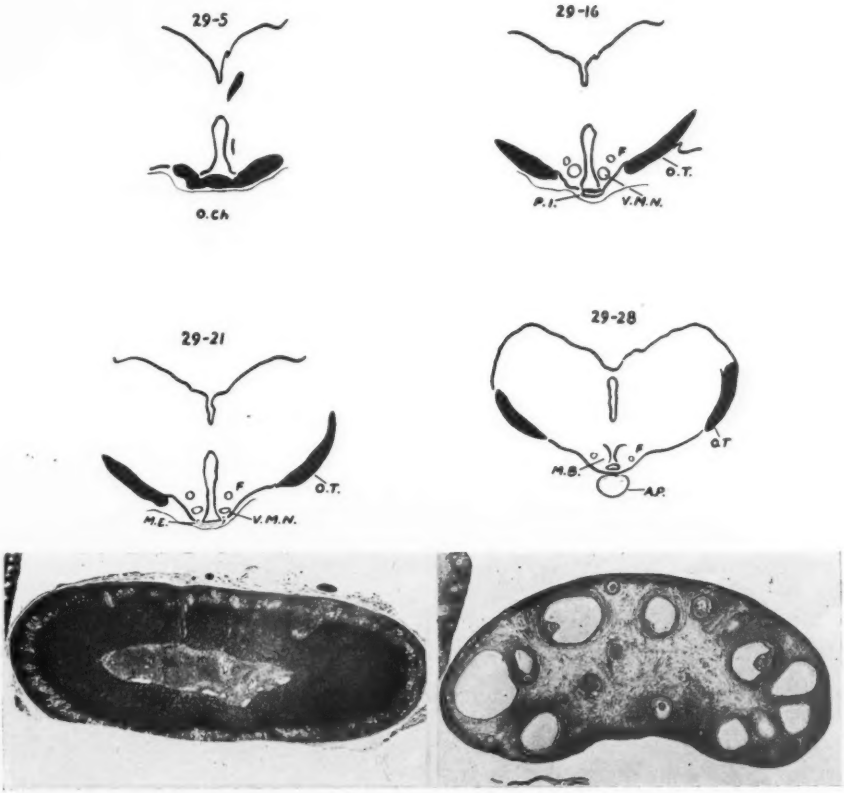

FIG. 1. Cat 29: Drawings of 4 hypothalamic levels illustrating suprachiasmatic type of lesion. The first number on top of each drawing identifies the animal, while the second indicates the number of the serial section. All solidly black areas represent sites of lesions, including those which are secondary to the lesions such as demyelinization of optic tracts at caudal levels. The abbreviations denote the following structures: A. P. Anterior pituitary gland, F. Fornix, M. B. Mammillary bodies, M. E. Median eminence, or infundibulum, P. I. Pars infundibularis of anterior pituitary gland, O. Ch. Optic chiasm, O. T. Optic tracts, V. M. N. Ventromedial hypothalamic nucleus. In lower left, adrenal stained with hematoxylin and eosin. Light cortical areas are sites of remaining lipid deposits. Adrenal steroid output: 1.09 μg./min. Right lower field shows normal ovary.

able lipids of the adrenal cortex (Figs. 1 and 2). These findings were essentially the same as those in a group of control cats (84, 65, 66) without hypothalamic lesions. One control cat (83) responded to stress with a low

output of adrenal steroid. Histological examination of the adrenal glands in this cat revealed extensive cortical calcification. Between masses of calcific deposits, small groups of lipid-laden cortical cells were seen. This observation illustrates the importance of ruling out local disease as a cause of altered functional activity of the adrenal cortex.

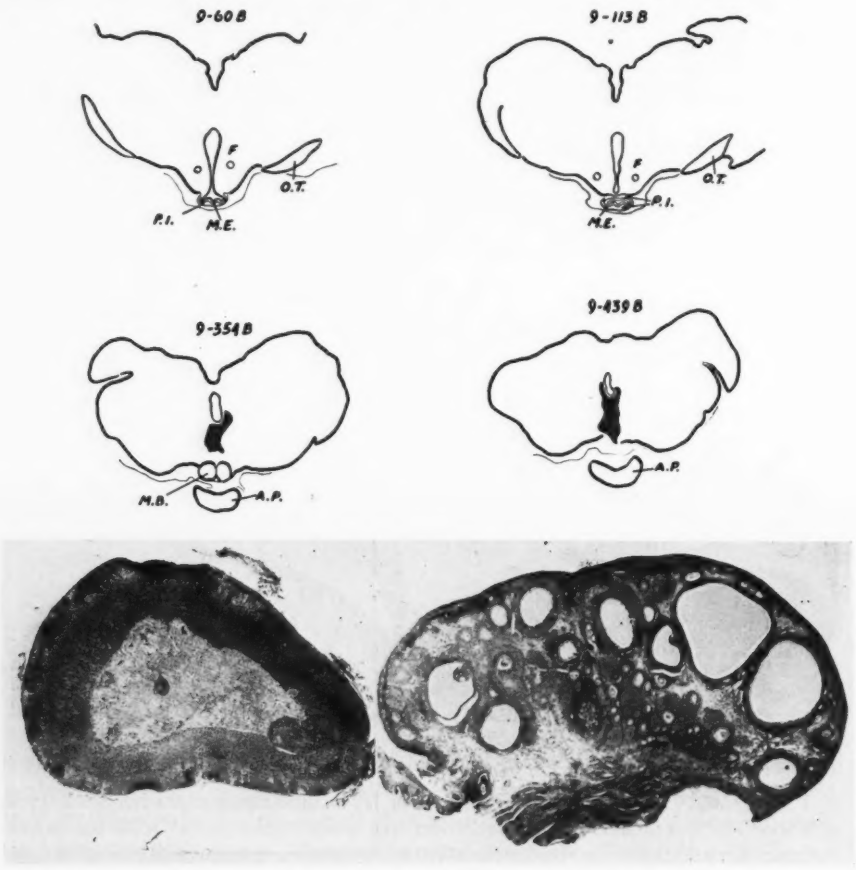

FIG. 2. Cat 9: Drawings of 4 hypothalamic levels illustrating destruction of posterior halves of the mammillary bodies. Adrenal cortex in left lower field shows severe lipid depletion. Adrenal steroid output: 0.85 μg./min. Normal ovary in right lower field. See legend of Fig. 1 for abbreviations.

In contrast to the control animals and those with lesions in the suprachiasmatic or posterior areas, the animals with lesions destroying the region of the median eminence showed very little response to the same degree of stress. The adrenal steroid output of the animals in the latter group was reduced to very low levels, averaging 0.37 μg. per minute. Again, the

morphological findings correlated well with the hormonal titer (Fig. 3). The adrenals had a broad subglomerular lipid zone which at autopsy could be recognized as a distinct yellow band. Histologically, the outer fascicular zone was composed of cells which were filled with single or multiple large fatty droplets. The lipid zone accounted for one-third to one-half of the entire thickness of the fascicular layer.

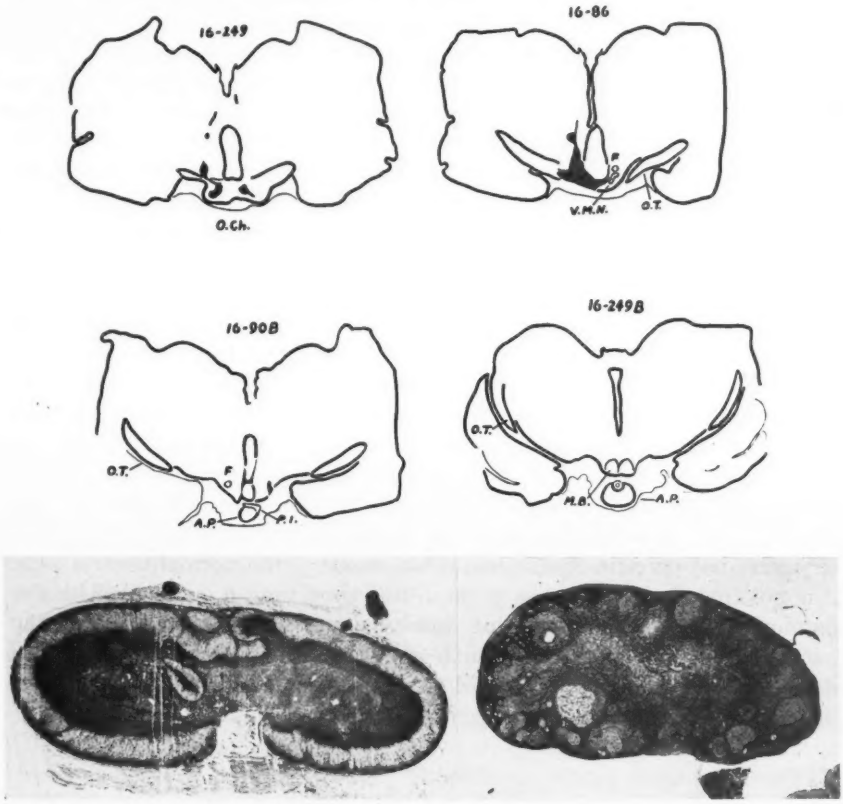

FIG. 3. Cat 16: Drawings of 4 hypothalamic levels, showing median eminence lesion extending rostrally into posterior part of chiasm and caudally to the region in front of mammillary bodies. Adrenal in left lower field shows broad lipid-laden outer fascicular zone. Adrenal steroid output: 0.25 μg./min. In right lower field, ovary with arrested follicular development. An organized corpus luteum is seen to the left. See legend of Fig. 1 for abbreviations.

Changes in the ovaries were also noted in the cats with lesions in the region of the median eminence (Table 1 and Fig. 3). The ovaries were reduced in size and contained small follicles, an occasional old corpus luteum and scattered stroma cells; large follicles were uniformly absent. By way of contrast, in all the experimental animals with lesions in the suprachias-

matic and posterior hypothalamic areas, ovarian follicular growth was normal.

Four of the nine animals with lesions in the region of the median eminence had anatomically intact anterior pituitary glands, as determined by serial sections. The remaining five cats had various degrees of anterior lobe damage. There was complete destruction of the anterior lobe in Cat 23 and subtotal destruction in Cat 20. Areas of atrophy and nodular hyperplasia were noted in Cat 61, while an anterior pituitary infarct of fairly recent age and involving the rostral portion of the gland was found in Cat 24. Microscopic studies of the anterior lobe cells after special staining procedures, such as the periodic acid Schiff reaction for beta cells and chrome-alum-hematoxylin-phloxin and trichrome stain for alpha and beta cells, failed to reveal distinct cellular abnormalities when comparison was made with similar preparations of the anterior lobes of the control group.

Polyuria occurred in nearly all cats during the first few postoperative days. Permanent polyuria was noted in the cats with lesions in the region of the median eminence except for two animals (Cats 23 and 20), already referred to, in which the anterior lobe of the pituitary had been totally or subtotally destroyed in the course of the production of lesions in the nearby hypothalamus. In addition, permanent polyuria was noted in Cat 76, in which the infundibular process had been destroyed by a lesion coextensive with the posterior hypothalamic area.

No changes were noted in the thyroid except in Cat 23, in which conspicuous atrophy had followed anterior lobe destruction. The thyroid glands of the cats subjected to hypothalamic and median eminence injury were not perceptibly different from those of the normal control cats. It is possible that differences existed, but they were not revealed by the histological methods, there being no obvious alteration in the size of the epithelial cells lining the acini, the degree of vascularity, or the appearance of the intra-acinar colloid. Nor did the histological examination of the anterior lobes yield evidence to suggest a hypothyroid state.

DISCUSSION

The data presented here clearly indicate that destruction of the median eminence and the adjoining hypothalamus interferes with the mechanism by which ACTH is released from the anterior lobe in response to an injection of epinephrine or to a stressful stimulus such as laparotomy. These data also show that destruction of the mammillary bodies does not disturb this mechanism, which is in contrast to the findings of De Groot and Harris (1950, 1952) and of Porter (1953) who have reported that lesions in the posterior hypothalamus abolish the ACTH response to stress. The criteria for judging ACTH-adrenal activity which were used by these authors, namely lymphocytopenia and eosinopenia, may account for the difference in findings from those reported here. Lesions in the suprachiasmatic area, also have no disturbing effect.

Although an intact median eminence is necessary for the release of ACTH in response to certain stimuli, there is evidence from these data that the adrenocorticotrophic function of the anterior hypophysis is not completely under the control of the hypothalamus. Destroying the median eminence does not cause the adrenal cortex to atrophy such as is seen when the anterior lobe is removed. Furthermore, the animals with the median eminence destroyed did not show obvious signs of hypoadrenocortical function. It would seem, therefore, that it is the acute release of ACTH in response to a stressful stimulus which is subject of hypothalamic control. These observations give no clue as to the nature of the mechanism by which the median eminence influences the anterior hypophysis.

In contrast to the finding with ACTH the gonadotrophic hormones are not secreted in amounts necessary to maintain a normal ovary if the median eminence and the adjoining hypothalamus are destroyed, judging from the failure of follicular growth of the ovaries in the cats with lesions in this area. It would seem therefore that the dependence of the gonadotrophic function of the anterior hypophysis upon the hypothalamus is greater than that of adrenocorticotrophic function.

SUMMARY

Studies are reported on 17 cats in which lesions were made in the hypothalamus with the Horsley-Clark stereotaxic instrument, and on 4 cats without hypothalamic lesions. Cats with lesions in the suprachiasmatic area and in the posterior area of the hypothalamus responded to stressful stimuli such as an injection of epinephrine and to laparotomy as did normal cats, with a marked decrease in eosinophiles and with an outpouring of adrenal steroids ranging from 0.85–1.42 μg. per minute. Correlated with this was a depletion of stainable lipids in the fascicular layer of the adrenal cortex. The ovaries showed normal follicular growth.

The animals with lesions in the median eminence and the adjacent hypothalamus showed very little response to similar stressful stimuli. There was a decrease in the epinephrine-induced eosinopenia. The eosinopenic response to laparotomy was retained but in an attenuated form. The adrenal steroid output was reduced to amounts which were not detectable or to low levels ranging between 0.02 and 0.92 μg. per minute. The adrenal cortex of such animals showed large amounts of stainable lipids in the fascicular layer. The ovaries showed arrested follicular growth. Diabetes insipidus was present in this group of animals.

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DIABETOGENIC ACTION OF PROLACTIN

B. A. HOUSSAY, E. ANDERSON, R. W. BATES AND CHOH HAO LI

(Institute of Biology and Experimental Medicine, Costa Rica 4185, Buenos Aires; National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland; University of California, Berkeley, California)

IT HAS been difficult to establish whether the effects on carbohydrate metabolism of different preparations of prolactin are due to the hormone itself or to other pituitary hormones which may be present in small amounts in these preparations. Thus, the clearly marked anti-insulin effect of impure pituitary preparations (Young, 1936; Marks, 1936) is reduced so as to be infinitesimal in purified preparations (Young, 1948, b, c). Riddle and his associates, in several papers published between 1937 and 1947, reported that prolactin had hyperglycemic and diabetogenic activity; later they (Riddle *et al.*, 1947) found that prolactin preparations which were free of adrenocorticotropin (ACTH) did not modify the blood sugar of the fasting pigeon, and the slight increase in blood sugar observed in animals which were fed seemed to be due to a greater ingestion of food. Hepatic glycogen, however, was found to be increased by prolactin in normal pigeons, and even more so in hypophysectomized animals. Also, the beta cells of the islets of Langerhans at first lost their granules, and later showed stimulation (Riddle *et al.*, 1947).

Prolactin preparations which contained ACTH increased glycosuria in hypophysectomized-pancreatectomized cats, but not in adrenalectomized-pancreatectomized cats (Long, 1937). Barnes observed the glycosuric action of prolactin in pancreatectomized dogs in which glycosuria had been controlled by diet (Riddle and Bates, 1937). High doses of prolactin given over a long period had no diabetogenic or anti-insulin effect in rabbits or monkeys (Young, 1938, a, b); some of the rabbits, however, had a transitory glycosuria (Young, 1938, a). Two hypophysectomized dogs with partial pancreatectomy received 12 mg. prolactin daily during 4 days; in one there was no change in the blood sugar, in the other it rose from 97 to 130 mg.% (Houssay and Biasotti, 1938).

Much purer preparations of prolactin had a diabetogenic effect in hypo-

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physectomized-pancreatectomized toads (A. Houssay, 1945; Houssay and Anderson, 1949). Squibb's 71713 preparation of prolactin, prepared by Bates, had diabetogenic activity in dogs (Houssay and Anderson, 1949; Houssay and Rodriguez, 1953) and cats (Houssay and Anderson, 1949), made more sensitive by partial pancreatectomy. This same preparation (71713) injected intramuscularly in doses of 3 to 5 mg./kg. daily reduced the insulin sensitivity of hypophysectomized dogs, and decreased or suppressed the effect of insulin in hypophysectomized-adrenalectomized dogs, it also reduced secondary hypoglycemia following the administration of glucose (De Bodo and Sinkoff, 1953; De Bodo, Sinkoff, Den and Kiang, 1953; Sinkoff and De Bodo, 1953).

In dogs in which the pancreas had been reduced somatotrophin had the greatest diabetogenic activity, prolactin had a lesser effect and ACTH the least (Houssay and Anderson, 1949). Unnecessarily high doses, however, were used, because the object of that work was to demonstrate which hormones had diabetogenic action. Later, smaller doses were found to be effective in partially pancreatectomized dogs with thyroid intact or after thyroidectomy (Houssay, Rodriguez and Cardeza, 1953). The preparation used in this work (Squibb's 71713) had small amounts of corticotrophin, therefore further experiments have been performed with pure prolactin prepared by Li.

PLAN AND METHODS

Dogs and cats were used, after reducing their pancreatic tissue in order to increase their sensitiveness to hypophyseal hormones and to obtain diabetogenic effects with much smaller doses. Seventy-seven to 87% of the pancreas was removed in dogs (Tables 1 and 2) and 61–87% was removed in cats. During the first 4 to 5 days after operation the animals received 0.3–0.5 u/kg. of insulin in order to protect the remaining pancreatic tissue, to prevent possible hyperglycemia or glycosuria and to facilitate healing of the wound. The thyroids were removed in 2 of these animals, leaving the parathyroids, and in another the hypophysis was removed. The animals were used 33 to 45 days after pancreatectomy, except in two cases in which the experiments were begun 16 and 18 days after the operation.

The hormone, in 1 to 3 ml. of 0.8% saline, was injected intraperitoneally in 4 dogs and all the cats and subcutaneously in the other dogs. Blood sugar determinations were made, on blood obtained from the ear, by the Haslewood-Strookman method in 3 dogs and 2 cats and by the Somogyi-Nelson method in all the other animals. Toward the end of the morning the animals were fed raw beef or horse meat, bread and milk.

A given dose of hormone was injected daily during a 4-day period. This was administered immediately after drawing the blood for sugar determination. Treatment was then interrupted and 7 to 10 days later, after the blood sugar had returned to normal, treatment was again started with a different dose of the same hormone, or with another hormone. Blood sugar was considered to have reached a diabetic level when the morning blood sugar was 150 mg.% or higher, after an 18 hour fast.

Experiments were also made on the toad *Bufo arenarum* Hensel and in *Leptodactylus occelatus* L. Gir, in which the pancreas and the *pars distalis* of the hypophysis had been removed. The hormone was injected under the skin and blood was drawn from the heart on the following day for blood sugar determination.

Three preparations of prolactin were used. The first one prepared by C. H. Li, was used in toads in 1945. The second was #71713 prepared by R. W. Bates and used by Houssay and Anderson (1949), De Bodo *et al.* (1953) and Houssay, Rodriguez and Cardeza (1953). The third is a preparation of pure lactogenic hormone of sheep's hypophysis (Cole and Li, 1954).

The following are the characteristics of prolactin 71713 prepared and assayed by Bates:

	Potency	Activity of pure hormone	Content
Prolactin	20–25 I.U. mg.	25 I.U./mg.	80–100%
ACTH	0.02–0.05 U.S.P. U./mg.*	100–200 U.S.P. U./mg.	<0.1%
TSH	0.02 U.S.P. U./mg.†	(10?) U.S.P. U./mg.	<1%
LH and FSH	1%	?	1%
Growth	<1%‡	?	<1%

* Ascorbic acid depletion, intravenous, hypophysectomized rat.
† Fixed iodine depletion, subcutaneous, chick thyroid.
‡ Negative in hypophysectomized rats with 10 mg./day.

The prolactin prepared by Li was obtained from sheep's hypophysis by a method involving counter-current distribution (Cole and Li, 1954). It contained 35 I.U. per mg. and was essentially free from other contaminants. It behaved as a pure protein based on the following criteria: N-terminal amino acid analysis, counter-current distribution, the analysis of amino acid sequence and composition. A total dose of 14 mg. injected into immature 30 day old female rats, 7 days after hypophysectomy showed no thyrotrophic, gonadotrophic or adrenocorticotrophic effect. According to tests made by the ascorbic acid depletion method it was estimated that 200 μg. of the preparation was contaminated with the equivalent of 0.1 μg. α-corticotrophin. A total dose of 10 mg. given over 4 days did not increase the width of the tibial cartilage, therefore, it was considered to be free from growth hormone. In the hypophysectomized *Rana pipiens* a dose of 25 μg. had no intermedin activity.

RESULTS

In dogs with an intact pancreas no hyperglycemia was observed when 5 mg./kg. of prolactin was injected daily for 4 days. One cat (Table 3) with an intact pancreas did not have hyperglycemia after receiving 25 mg. (13.8 mg./kg.)daily during 3 days and 50 mg. (27.5 mg./kg.) daily during 2 days.

Dogs with reduced pancreas. Dogs of both sexes with a reduced pancreas became diabetic when injected with Bates' 71713 prolactin or Li's pure prolactin (Tables 1, 2 and 4). Fifteen treatments made in 11 dogs gave the following results: with 1 mg./kg. per day a diabetic response was obtained 4 times and was absent twice; with 2 mg./kg. it was obtained twice and was absent twice; with 4.5 to 5.7 mg. it was obtained 3 times and not in 2 others.

Of the 11 dogs injected 7 became diabetic. There was an increase in blood sugar, but not up to the 150 mg. % level in 3, and there was no increase in one. All of the 5 males and 2 of the females became diabetic. Female dogs 10 and 30 secreted milk in large amounts during treatment with prolactin. Dog 30 was diabetic, dog 10 had no hyperglycemia.

TABLE 1. DIABETOGENIC ACTIVITY OF PROLACTIN 71713 IN DOGS WITH PARTIAL PANCREATECTOMY

Dog No.	Weight, kg.	Sex	Pancreas remaining %	Prolactin, mg./kg./day	Blood sugar, mg. % (1)	Blood sugar, mg. % (2)	Somatotrophin, mg./kg./day	Blood sugar, mg. % (1)	Blood sugar, mg. % (2)	ACTH, mg./kg./day	Blood sugar, mg. % (1)	Blood sugar, mg. % (2)
Partial Pancreatectomy												
2B*	10.9	f	13	5.7	74	147	5.7	75	296	5.7	74	86
5B*	11.0	f	16	5.0	72	134	5.0	71	185	5.0	79	89
30B*	9.1	f	14	5.5	65	168	5.5	67	336	5.5	74	140
10B*	11.0	f	14	4.5	68	133	4.5	65	90	4.5	80	84
5B†	13.0	m	20	1.0	74	170	1.0	75	373	—	—	
Partial pancreatectomy, total thyroidectomy												
12†	8.4	f	23	1.0	71	96	1.0	102	316	—	—	
18†	9.0	m	22	1.0	93	233	1.0	81	358	—	—	
Partial pancreatectomy, hypophysectomy												
13†	6.8	f	16	1.0	99	249	1.0	99	247	—	—	

(1) Initial fasting blood sugar.
(2) Maximal fasting blood sugar during treatment.
* Injected intraperitoneally for 4 days.
† Injected subcutaneously for 4 days.

The hormone was given in large doses to the first 4 dogs, because it was not then known that it had diabetogenic activity. In the other 7 dogs smaller doses were tried and found to be effective.

The diabetogenic activity of prolactin was definitely below that of somatotrophin in 5 cases, higher in one case, and in another both hormones produced the same effect. Prolactin was more potent than ACTH in all cases (Houssay and Anderson, 1949).

The rise in blood sugar is usually evident by the 3rd or 4th day of treatment; occasionally, it appears on the 2nd or 5th day (Table 4). Diabetes was always transitory; however, treatment was not continued in an effort to obtain permanent diabetes.

The following signs were observed in dogs: (a) *hyperglycemia* (Tables 1, 2 and 4); (b) *glycosuria* in relation to the increase in blood sugar; (c) *polyuria*, when there was intense hyperglycemia (up to 350–660 ml. per day); (d) *polydipsia;* (e) *polyphagia;* (f) *ketonuria*. The thyroid and adeno-

TABLE 2. DIABETOGENIC ACTIVITY OF LI'S 1953 PROLACTIN IN DOGS WITH PARTIAL PANCREATECTOMY

Dog no.	Weight, kg.	Sex	Pancreas remaining %	Prolactin, mg./kg./day	Fasting blood sugar	
					initial, mg. %	maximal, mg. %
36	6.8	m	21	1	80	128
				2	95	178
				5	90	237
37	11.0	m	21	1	94	190
39	8.0	m	14	2	101	168

TABLE 3. DIABETOGENIC ACTIVITY OF PROLACTIN 71713 IN CATS

Cat no.	Weight, kg.	Sex	Pancreas remaining %	Prolactin,* mg./kg./day	Blood sugar, mg. % (1)	Blood sugar, mg. % (2)	Somatotrophin,* mg./kg./day	Blood sugar, mg. % (1)	Blood sugar, mg. % (2)
					Partial pancreatectomy				
1	3.4	f	25	7.3	89	97	7.3	82	304
3	2.3	m	27	10.7	82	221	10.7	99	278
5	4.2	m	33	5.9	78	190	—	—	
6	3.1	m	39	7.4	84	203	—	—	
				7.4	80	107			
7	2.3	f	29	10.8	74	167	—	—	
				10.8	76	151			
8	3.6	m	13	6.9	74	110	—	—	
9	2.3	f	26	10.7	80	210	—	—	
				10.7	83	213			
11	1.6	f	37	15.6	79	212	—	—	
				31.2					
					Intact pancreas				
10	1.8	m	100	13.8	78	80	—	—	
				27.5	76	78			

* Injected intraperitoneally on 4 consecutive days.
(1) Initial fasting blood sugar.
(2) Maximal fasting blood sugar during treatment.

hypophysis are not necessary for the diabetogenic effect of prolactin (Tables 1 and 4).

Cats. Prolactin preparation #71713 was injected into 8 cats with a surgi-

TABLE 4. ACTION OF PROLACTIN ON THE BLOOD SUGAR OF DOGS

Dog no.	Prolactin	Doses, mg./kg./day	No. days injected	No. days from pancreatectomy	Blood sugar, mg. %							
					Days							
					Before	1	2	3	4	5	6	7
					Partial pancreatectomy							
2B	71713	5.7	4	57	74	*69*	*66*	*68*	*109*	147	83	66
5B	71713	5.0	4	59	72	*76*	*76*	*83*	*101*	134	—	66
30B	71713	5.5	4	28	65	*64*	*89*	*95*	*115*	168	115	109
5	71713	1	3	96	74	*73*	*170*	*99*	78	—	—	—
					Partial pancreatectomy and thyroidectomy							
12	71713	1	4	26	71	*71*	*82*	*70*	*96*	—	—	—
18	71713	1	4	32	93	*108*	*93*	*105*	*233*	74	—	—
					Partial pancreatectomy and hypophysectomy							
13	71713	1	4	74	99	*116*	*137*	*127*	*197*	249	100	99
					Partial pancreatectomy							
36	Li	1	5	52	80	*128*	*102*	*108*	*113*	—	90	—
	Li	2	4	27	95	*110*	*178*	*143*	*170*	178	—	—
	Li	5	4	32	90	*118*	*143*	*237*	*210*	—	—	112
37	Li	1	5	16	94	*125*	*117*	*177*	*190*	*122*	—	87
39	Li	2	4	19	101	*101*	*135*	*158*	*168*	—	—	—

TABLE 5. ACTION OF PROLACTIN 71713 ON BLOOD SUGAR OF CATS WITH PARTIAL PANCREATECTOMY

Cat no.	Prolactin, mg./kg./day	Days injected	Days after pancreatectomy	Blood sugar, mg. %						
				Days						
				Before	1	2	3	4	5	6
1	7.3	3	14	89	*89*	*73*	—	80	—	—
3	10.7	3	12	82	*102*	*113.*	*221*	—	—	310
5	5.9	4	45	102	*140*	*181*	*190*	*149*	152	—
6	7.4	4	21	80	*83*	*92*	*105*	*158*	203	97
6	7.4	4	34	90	*89*	*98*	*107*	*96*	105	90
7	10.8	4	10	80	*87*	*126*	*159*	*162*	167	129
4*	10.8	4	32	94	*98*	*103*	*119*	*151*	183	102
8	6.9	4	37	105	*102*	*108*	*110*	*110*	96	—
11	15.6 31.2	3	111	90	*120*	*105*	*112*	*159*	*212*	—

* During treatment this animal received 1 unit insulin daily.

cally reduced pancreas and into one with a normal pancreas (Tables 3 and 5). Two animals with a reduced pancreas (cats 1 and 3) received 25 mg. prolactin daily, i.e., 5.9 to 10 mg./kg. per day, during 3 days; five (cats 5, 6, 7, 8 and 9) received the same dose during 4 days, two of these (cats 6 and 7) on two occasions. Cat 11 received 25 mg. (15.6 mg./kg.) daily during 3 days, and 50 mg. during 3 days.

Seven out of the 8 partially depancreatectomized cats became diabetic; only cat 8 failed to show diabetes. Cat 6 had hyperglycemia in the course of the first treatment, but not when treated a second time. Cats 7 and 9 had hyperglycemia both times they received treatment. Insulin (one unit per day) did not lower the bood sugar while prolactin was being injected (Table 5).

Cat 9 was given prolactin during 18 consecutive days in the course of the second treatment (25 mg. for 15 days and 50 mg. for 3 days). Blood sugar rose to 213 mg.% on the 4th day and remained high for 3 days; during the second week the blood sugar fluctuated between 96 and 195 mg. (during 4

TABLE 6. ACTION OF PROLACTIN, GROWTH HORMONE AND ACTH ON BLOOD SUGAR OF HYPOPHYSECTOMIZED-PANCREATECTOMIZED TOADS *Bufo Arenarum* HENSEL

Dose per animal, mg.	Blood sugar, mg. %			
	Growth hormone	ACTH	Prolactin	Uninjected controls
1.6	141	93	108*	56
1.6	137	107	107*	52
1.0	144	163	70†	58
1.0	119	138	102†	50
1.0	110	80	88†	77
1.6			72‡	76

Each blood sugar value is the average for a group of 10 to 20 toads.
Hormone was injected at time of operation and blood sugar determined 24 hours later.
* Li's prolactin 1945.
† Bates' prolactin 71713.
‡ Li's prolactin 1953.

days it was 124 to 141 mg.%); during the third week it decreased and remained between 90 and 122 mg.%. Apparently there was some degree of habituation.

The following signs were observed in the cats during the diabetic stage: (a) *hyperglycemia* (Table 3); (b) *glycosuria*, 2 to 20 g. per 100 ml., 1 to 4 g. per kg. per day; (c) *polyuria* (100 to 230 ml. per day); (d) *polyphagia*; (e) *polydipsia*; (f) *ketonuria* (4.9 to 45 mg. per kg. per day).

Toads. Toads (*Bufo aernarum* Hensel) were injected with prolactin or

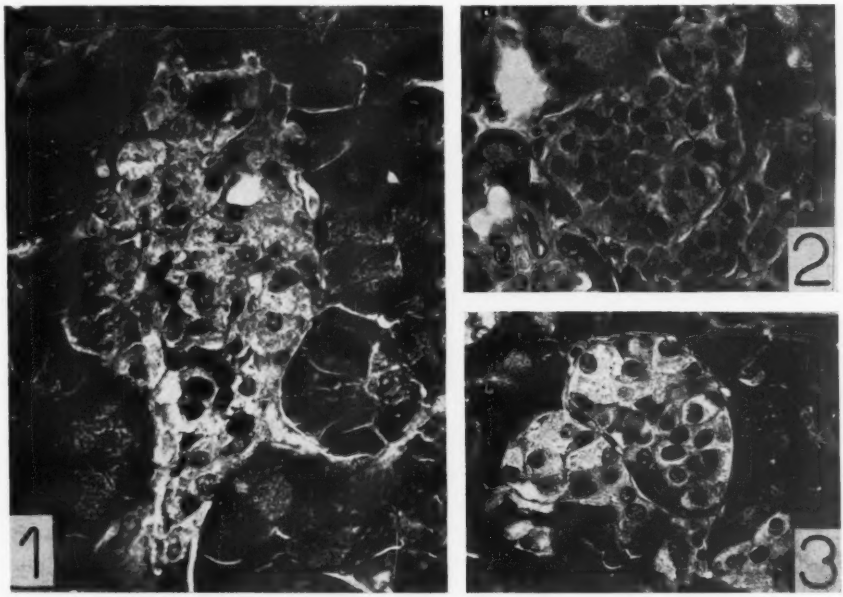

FIG. 1. Dog 36, Diabetes produced by prolactin prepared by Li (Table 2). Small islets, diminution of β cells in relation to α cells. Degranulation and vacuolization of β cells.

FIG. 2. Cat 11, Langerhans islets, before treatment.

FIG. 3. Cat 11, Langerhans islets, prolactin diabetes. Degranulation and hydropic degeneration (glycogen) of β cells.

other hormones after removal of the *pars distalis* and the pancreas (Table 6). Prolactin had a slight diabetogenic effect, statistically significant in 3 out of 6 experiments. Somatotrophin, on the other hand, was always more active when given in the same dose as prolactin. ACTH was more active than prolactin in 2 experiments.

Histological findings. Histological examination of the pancreas in the cases examined showed changes in the β-cells following treatment with prolactin (Figs. 1–3). There was a diminution of β cells as compared to α cells and degranulation and vacuolar or hydropic degeneration of β cells.

DISCUSSION

The diabetogenic activity of prolactin is not as great as that of somatotrophin, and may pass unnoticed if very sensitive animals are not used. In the experiments reported in this paper a large part of the pancreatic tissue was removed in order to increase sensitiveness of diabetogenic factors. Dogs and cats are sensitive to prolactin.

There was no detectable somatotrophin in the prolactin preparations used; 71713 had a small amount of ACTH (0.1%), but its effect on the dog was greater than that of ACTH alone (Table 1). Li's prolactin contained about 0.05% ACTH, yet it was very active in the dog. A preparation of prolactin completely free from ACTH would be necessary in order to exclude all possibility of ACTH potentiating the diabetogenic activity of prolactin.

Diabetogenic activity may be seen after a second treatment with prolactin (Tables 2 and 3). Large doses usually have greater effects than small ones (Table 2). Diabetogenic activity has been demonstrated in well-fed animals; the effect of fasting has not been tested.

These experiments seem to show that prolactin is one of the diabetogenic hormones of the *pars distalis* of the hypophysis. So far, somatotrophin, ACTH and prolactin have been demonstrated as having diabetogenic activity. Prolactin is less potent than somatotrophin with respect to this activity.

SUMMARY AND CONCLUSIONS

Prolactin had a diabetogenic effect in dogs and cats in which the pancreatic tissue had been reduced surgically in order to increase their sensitiveness to diabetogenic factors. Prolactin also showed diabetogenic activity in hypophysectomized-pancreatectomized toads, but not in all cases. The diabetogenic activity seems to be due to prolactin or some other substance that accompanies it in preparations free from somatotrophin and with very little ACTH. In partially pancreatectomized dogs the diabetogenic activity of prolactin was much weaker than that of the same dose of somatotrophin, but greater than that of ACTH.

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INFLUENCE OF EPINEPHRINE AND CORTISONE ON THE METABOLISM OF THE HYPOPHYSIS AND HYPOTHALAMUS OF THE RAT[1]

SIDNEY ROBERTS AND MARTHA R. KELLER

The Departments of Physiological Chemistry and Anatomy, School of Medicine, University of California Medical Center, Los Angeles, and the Investigative Medicine Service, Veterans Administration Hospital, Long Beach, California

THE secretion of ACTH by the anterior pituitary gland is readily altered by a wide variety of non-specific stresses and by the administration of physiologic agents such as epinephrine and the adrenal cortical steroids (Selye, 1946; Sayers, 1950; Long, 1952). It is presumed that these factors exert their effects primarily via the hypothalamico-hypophyseal pathway (Harris, 1948). No information is available, however, on possible changes in pituitary and hypothalamic metabolism which may accompany alterations in pituitary hormonal synthesis or release. In the present investigation, respiration and aerobic glycolysis in the anterior and posterior hypophysis and hypothalamus of the rat have been followed after non-specific stress and after the administration *in vivo* and *in vitro* of epinephrine and cortisone. The most striking effects observed were an increase in adenohypophyseal metabolism after *in vivo* or *in vitro* administration of epinephrine, and a decrease after cortisone treatment *in vivo*.

METHODS

The methods employed have been described in detail previously (Roberts and Keller, 1953). All incubations were carried out in micro-Warburg flasks of 5 ml. capacity, containing 0.5 ml. of Krebs-Ringer Phosphate medium with glucose added in a concentration equivalent to 75 mg. per 100 ml. Each flask contained six neurohypophyses, three adenohypophyses, or about 10 mg. of anterior or posterior hypothalamic tissue, obtained from adult male Wistar rats at sacrifice. The metabolism of these tissues was similar whether the animals were sacrificed by decapitation or under pentobarbital sodium (Nembutal) anesthesia. The latter procedure was therefore employed uniformly in these studies. Substances added *in vitro* were suspended or dissolved in 0.05 ml. of medium and were tipped in from the side-arm after one hour of incubation. Cortisone acetate and hydrocortisone acetate (Merck) were added as a suspension, employing a minute quantity of "Tween 80" as the dispersing agent. Epinephrine (U.S.P.) was added in solution, in some instances with 1×10^{-4}M. glutathione present to prevent rapid oxidation. Control

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incubations were carried out in which "Tween 80" or glutathione alone were added from the side-arm. The results in these latter instances were not different from those obtained in the absence of these materials.

RESULTS AND DISCUSSION

Effects of Epinephrine in Vivo and in Vitro

Figure 1 illustrates the influence of epinephrine injected intraperitoneally (20 μg. per 100 gm. body wt.) on respiration and aerobic glycolysis in the anterior and posterior hypophysis and hypothalamus of the rat. A significant increase in oxygen consumption and glucose utilization *in vitro* was noted in anterior pituitary and posterior hypothalamic tissue 20 hours after the administration of epinephrine. No change in lactate accumulation was evident in these tissues. A decline in respiration and aerobic glycolysis was observed in the posterior pituitary gland. No *significant* change occurred in the metabolism of the anterior portion of hypothalamus over the 20 hour time interval. Non-specific stresses including exposure to cold for 2 or 16 hours or the injection of formaldehyde (0.3 ml. of a 10% solution) produced no significant alterations in hypophyseal or hypothalamic metabolism.

TABLE 1. IN VITRO EFFECTS OF EPINEPHRINE AND CORTISONE ON METABOLISM OF RAT ANTERIOR PITUITARY GLANDS

Hormone added* (μg.)	No. of expts.	Oxygen consumption†		Glucose disappearance†	Lactate accumulation†
		1st hr.	2nd hr.		
		(μl./mg. wet tissue)		(μg./mg. wet tissue/3 hrs.)	
None	56	1.19±0.02	1.16±0.03	5.7±0.2	3.8±0.1
Epinephrine, 0.004	8	1.21±0.03	1.18±0.04	5.9±0.3	4.0±0.2
Epinephrine, 0.1	15	1.22±0.06	1.65±0.05	6.8±0.5	4.2±0.2
Epinephrine, 0.2	19	1.13±0.05	1.73±0.04	8.3±0.3	4.1±0.3
Epinephrine, 1.0	7	1.23±0.03	1.00±0.06	6.1±0.3	3.6±0.2
Cortisone, 10	8	1.30±0.04	1.29±0.05	5.7±0.4	3.7±0.2
Cortisone, 25	10	1.19±0.03	1.15±0.03	5.8±0.2	4.0±0.3
Cortisone, 250	9	1.25±0.04	0.55±0.07	3.8±0.3	3.1±0.2

* Hormone was tipped in from the side-arm after 1 hour. Incubation was then allowed to proceed 2 additional hours.
† Mean ± Standard error of the mean.

The addition of epinephrine (0.1 or 0.2 μg.) to the incubation flask also increased respiration and glucose utilization by normal rat anterior pituitary tissue *in vitro* (Table 1). The addition of smaller (0.004 μg.) or larger (1.0 μg.) amounts of the drug was without substantial effect on the metabolism of this tissue. No changes were noted in respiration or aerobic glycolysis of posterior pituitary tissue or hypothalamic tissue under similar circumstances (not shown).

It has been demonstrated repeatedly that epinephrine is capable of evoking the release of ACTH from the adenohypophysis (cf. Long, 1952). Available evidence indicates that this action of sympathomimetic sub-

stances may occur either as a consequence of activation of posterior hypothalamic-anterior hypophyseal neurohumoral pathways (Porter, 1954) or by direct stimulation of the adenohypophysis (McDermott *et al.*, 1950), or both. The present results are consistent with these observations. Thus,

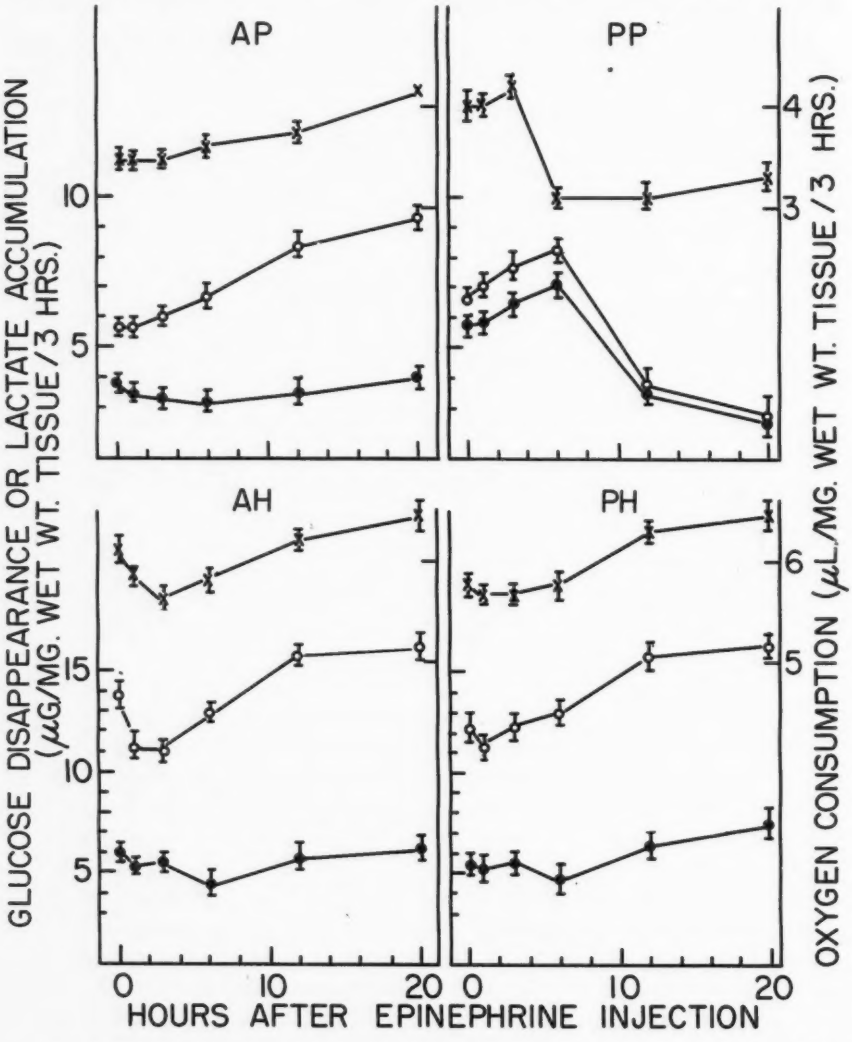

FIG. 1. The influence of epinephrine on respiration and aerobic glycolysis in anterior and posterior hypophyseal and hypothalamic tissue of the rat. Each point represents the mean ± the standard error of 7 to 56 experiments. Crosses, oxygen consumption; open circles, glucose disappearance; solid circles, lactate accumulation. AP, anterior pituitary; PP, posterior pituitary; AH, anterior hypothalamus; PH, posterior hypothalamus.

the accentuation in metabolism of adenohypophyseal and posterior hypothalamic tissue after *in vivo* administration of epinephrine suggests an activation of the neurohumoral pathway linking these structures during the release of ACTH. It would also appear that data indicating a local effect of epinephrine on the anterior pituitary gland has its counterpart in the *in vitro* stimulation of the metabolism of this tissue by epinephrine (Table 1).

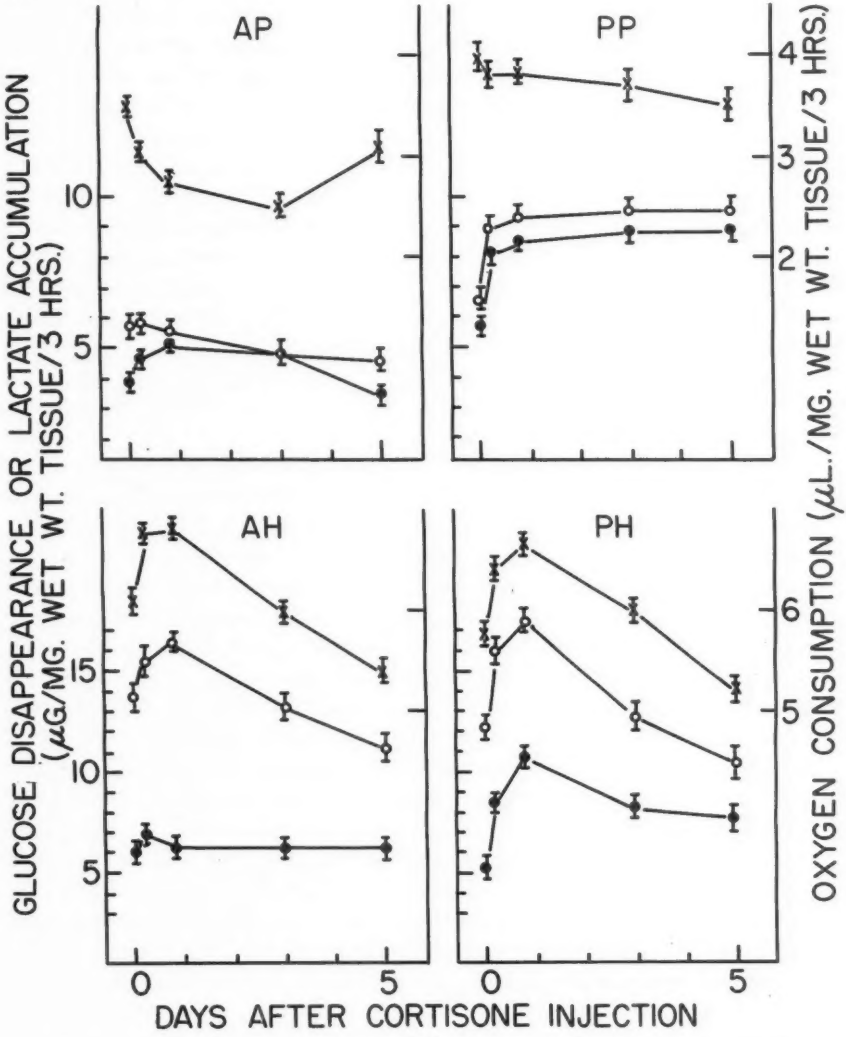

FIG. 2. The influence of cortisone on respiration and aerobic glycolysis in anterior and posterior hypophyseal and hypothalamic tissue of the rat. See the legend to Fig. 1 for explanations.

It should be pointed out that the alterations observed in hypothalamic and hypophyseal metabolism in the present experiments are not necessarily *directly* associated with ACTH release. In fact, the secretion of this hormone is known to occur very rapidly after epinephrine administration (Vogt, 1944). It is possible that the changes noted are associated with hormone resynthesis rather than hormone secretion.

Effects of Cortisone in Vivo and in Vitro

The intravenous administration of cortisone (2.5 mg. per 100 gm. body wt.) produced a prompt decline in the oxygen consumption of surviving anterior pituitary tissue of the rat (Fig. 2). However, little or no change was noted in aerobic glycolysis. Respiration was significantly depressed within 4 hours after a single injection of this hormone and remained low for 3 days after daily injections of the same dose. Restoration of the initial levels occurred at 5 days even though hormone treatment was continued. Coincident with the rapid decrease in adenohypophyseal respiration following cortisone injection, an accentuation of respiration and aerobic glycolysis was noted in both anterior and posterior hypothalamic tissue. These effects were more striking in the posterior hypothalamus and disappeared within 3 days. Posterior pituitary tissue also exhibited an elevation of aerobic glycolysis 4 hours after cortisone injection which was sustained throughout the 5 day experimental period.

Moderate amounts (10 to 25 μg.) of cortisone acetate had no discernible effect on oxygen consumption and aerobic glycolysis when added *in vitro* to surviving anterior pituitary tissue (Table 1). Larger amounts (250 μg.) produced a depression in these values. However, the addition *in vitro* of 250 μg. of cortisone during incubation of posterior pituitary glands or anterior or posterior hypothalamus resulted in a comparable decline in respiration and glycolysis in these tissues as well. The *in vitro* inhibitory action of the large dose of cortisone would therefore appear to be of a nonspecific toxic nature. Similar results have been reported after the addition *in vitro* of adrenal steroids to surviving brain tissue (Gordan *et al.*, 1951).

Adrenalectomy produced no significant alteration in the metabolism of any of the tissues studied within 1 to 30 days after operation.

Cortisone administration *in vivo* has been shown to inhibit ACTH secretion by the adenohypophysis (Sayers and Sayers, 1949). This action is presumed to be mediated via the posterior hypothalamic region of the brain (cf. Porter, 1954). The accentuation in metabolism of the latter area noted in the present experiments concomitant with a depression in adenohypophyseal respiration suggests that cortisone may activate an inhibitory neurohumoral pathway between these two structures, rather than depress an excitatory one (cf., however, Porter, 1954).

The precise nature of the hypothalamic neurohumors presumed involved in the pituitary response to epinephrine and cortisone are at pres-

ent unknown. However, evidence has been presented for the existence of a lipide or lipoprotein factor which is elaborated specifically by the posterior hypothalamus and is capable of stimulating the release of ACTH by the adenohypophysis (Slusher and Roberts, 1954).

SUMMARY

Subcutaneous administration of epinephrine to the rat produced a significant elevation of respiration and aerobic glycolysis in anterior pituitary and posterior hypothalamic tissue within 20 hours after injection. Similar increases in the metabolism of adenohypophyseal tissue were produced by the addition of epinephrine *in vitro*. Cortisone, injected intravenously, reduced the oxygen consumption of anterior pituitary tissue within 4 hours. Concomitant increases in the respiration and aerobic glycolysis of posterior hypothalamic tissue were noted. Cortisone, added *in vitro*, depressed the metabolism of anterior and posterior hypophyseal and hypothalamic tissue, but only in large doses. It is suggested that both epinephrine and cortisone activate neurohumoral pathways in the posterior hypothalamico-adenohypophyseal system. The former appears to be of a stimulatory nature, while the latter is inhibitory. In addition, it appears that epinephrine is also capable of stimulating the anterior pituitary gland directly.

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ADRENAL CORTICAL EXTRACT ON THE OXYGEN CONSUMPTION OF PROPYLTHIOURACIL TREATED FROGS

THOMAS B. CALHOON

Department of Physiology, The Medical College of South Carolina, Charleston, South Carolina

STUDIES on the effect of adrenal cortical extract on the resting oxygen consumption of normal animals appear to be inconclusive, at least for homoiotherms. It has been reported that extracts of this gland cause a decrease (Marine and Bauman, 1922; Franke, 1932; Hill *et al.*, 1950), increase (Koehlar and Hastings, 1929), and no change (Harrop *et al.*, 1931; Webster *et al.*, 1932; Hitchcock *et al.*, 1938; Missiuro *et al.*, 1938) in oxygen consumption. On the other hand, studies carried out on poikilotherms, specifically *Carassius auratus* and *Rana pipiens* (Calhoon and Angerer, 1950 a, b, 1954) appear to give consistent results, adrenal cortical extract producing *ca.* 25–35% depression of resting oxygen consumption.

Because of the consistency of the effect of adrenal cortical extract on the resting oxygen consumption, it was assumed that this effect was due to a direct action on the tissues of the animal's body generally (Calhoon and Angerer, 1954). However, the degree of depression of oxygen consumption being the same order of magnitude as that which follows thyroidectomy, *ca.* 25–35% (Means, 1948), it was deemed possible that the depression observed might occur via an inhibition of thyroid activity. The purpose of this report is to determine if the second hypothesis is valid.

METHODS

Frogs (*Rana pipiens*) weighing 20 to 35 gm. were kept two weeks prior to use, in large concrete cages which were continuously bathed in running tap-water. The frogs were not fed during the course of the experiments.

Oxygen consumption determinations were made on three groups of animals: (a) one uninjected group, (b) one group injected with a suspension of 6-n-propylthiouracil in 0.65% saline, (c) one group injected with the propylthiouracil suspension plus adrenal cortical extract.

Oxygen consumption determinations were carried out weekly, by use of a direct-reading volumetric respirometer (Calhoon and Angerer, 1953). Metabolic rates were expressed as ml. of oxygen consumed/gm. wet weight/hour.

Injections of adrenal cortical extract were made subcutaneously in the abdominal region, while injections of the propylthiouracil were made via the dorsal lymph sac.

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RESULTS

Uninjected Controls

A group of 10 male frogs gave a mean oxygen consumption value of 0.156 ± 0.007 ml./gm. wet wt./hr. Thereafter the oxygen consumption fluctuated weekly, with extremes of *ca.* $\pm 15\%$ being evident, Figure 1, curve A. There was no trend away from the initial value for the duration of the experiment. The frogs, as expected continually lost weight so that at the end of the fifth week their mean body weight was *ca.* 15% below the

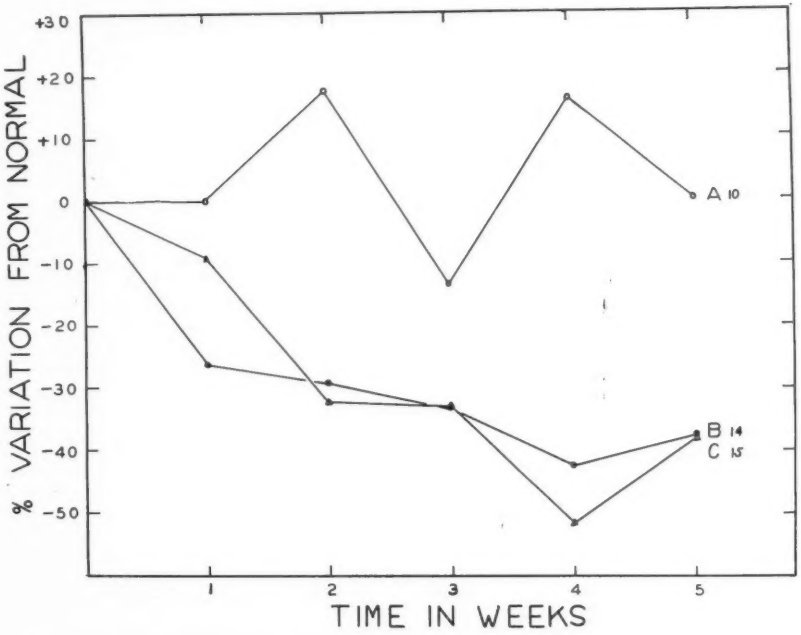

FIG. 1. Curve A shows the mean oxygen consumption of the uninjected control frogs. Curve B shows the effect of propylthiouracil on the mean oxygen consumption of frogs. Curve C shows the effect of propylthiouracil plus adrenal cortical extract on the mean oxygen consumption of frogs. The small numerals to the right of a given curve indicate the number of frogs used. Temperature: $30.00 \pm 0.1°$ C.

initial value, Figure 2, curve A. The lack of a drop of oxygen consumption in these frogs, probably reflects the fact that frogs are known to live for quite long periods of time without feeding, by using fat stores, in particular testicular fat (Calhoon and Angerer, 1954).

Injections of Propylthiouracil Suspension

A group of 14 male frogs gave a mean oxygen consumption value of 0.099 ± 0.004 ml./gm. wet wt./hr. These frogs were injected with powdered

propylthiouracil tablets[1] suspended in 0.65% saline. Propylthiouracil (0.033 mgm./gm. wet wt.) was given three times/week for a period of five weeks. Although this dose is *ca.* 8–9 times the dose usually given in clinical treatment on a gram-weight basis, there have been no comparative studies made between man and frogs. However, the dosage used was only one tenth of that previously reported in studies on the effects of thiourea in frogs (Joel *et al.*, 1949).

Oxygen consumption determinations were made once each week for the

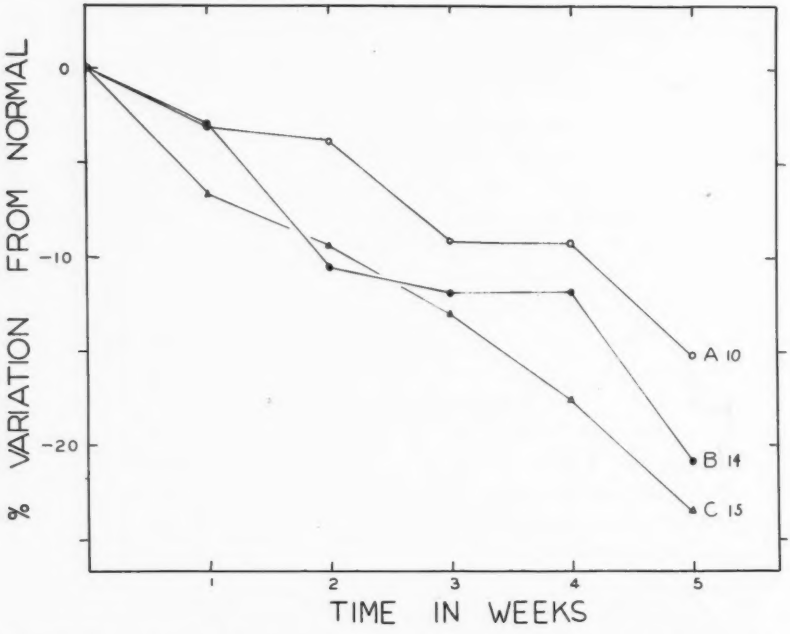

FIG. 2. Curve A shows the mean body weight change of the uninjected control frogs. Curve B shows the effect of propylthiouracil on the mean body weight of frogs. Curve C shows the effect of propylthiouracil plus adrenal cortical extract on the mean body weight of frogs. The small numerals to the right of a given curve indicate the number of frogs used.

five weeks, and the mean values are shown in Figure 1, curve B. This curve shows that the mean oxygen consumption was depressed exponentially with time, reaching a value of *ca.* 40% below the initial value. All mean values are statistically significant (Student's method) when compared to their own initial uninjected value, or to the values for the uninjected frogs.

Since these frogs were not fed they continually lost weight, so that at the

[1] Supplied through the Courtesy of The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana.

end of the fifth week, their mean body weight was *ca.* 21% below the initial mean body weight, Figure 2, curve B. There is no significance between the weight loss of these frogs and the uninjected control group ($P > 0.6$).

Injection of Propylthiouracil plus Adrenal Cortical Extract

A group of 15 male frogs gave a mean oxygen consumption value of 0.109 ± 0.008 ml./gm. wet wt./hr. These frogs were injected with 0.033 mgm. of propylthiouracil/gm. wet wt., and 0.00036 ml. of Upjohn's Adrenal Cortical Extract/gm. wet wt. three times/week, for the 5-week period.

Oxygen consumption determinations were again made once each week for the 5-week period, and the mean values of this group of frogs exhibited a depression, reaching a low of *ca.* 45% below the initial value, Figure 1, Curve C. All mean values, with the exception of that for the first week, are statistically significant when compared to the initial uninjected value, or to the values for the uninjected control group. Curves B and C, Figure 1, are not statistically different at any week with the possible exception of the first week ($P = 0.10$–0.05).

At the end of the fifth week, the mean body weight of this group of frogs was *ca.* 24% below their initial mean body weight, Figure 2, Curve C. There is no significance between the weight loss of these frogs and the uninjected control group ($P = 0.6$).

DISCUSSION

While the oxygen consumption of normal frogs remains relatively constant for the period tested, frogs given propylthiouracil only, and frogs given propylthiouracil plus adrenal cortical extract exhibit a marked depression of oxygen consumption (*ca.* 45%). The depression of oxygen consumption produced by adrenal cortical extract alone (Calhoon and Angerer, 1954) is of the same order of magnitude, duration and rate, as that produced by propylthiouracil given alone. Further, the depression of metabolism produced by simultaneous injections of propylthiouracil and adrenal cortical extract is no greater than that produced by propylthiouracil when given alone, Figure 1, Curves B and C. This suggests that the depression of oxygen consumption observed in frogs given adrenal cortical extract alone is due to an inhibition of thyroid activity.

Although no direct evidence exists for the frog that depression of the output of one trophic hormone (ACTH), leads concomitantly to a depression of the output of other trophic hormones of the anterior pituitary gland (thyrotrophin), there does exist a pituitary-adrenal axis (Atwell, 1935, 1937) as well as a pituitary-thyroid axis (Joel *et al.*, 1949) in the frog. Indeed, these same events have been observed in homoiotherms, specifically, man. It has been reported (Hardy *et al.*, 1950; Hill *et al.*, 1950) that ACTH as well as cortisone causes a depression of I^{131} uptake by the

thyroid, a decrease of blood protein-bound iodine by 50%, and a decrease of metabolic rate for long periods of time. This is analogous to other observations that testosterone propionate (Rubinstein *et al.*, 1939) and estrogens (Bugbee and Simonds, 1926) depress somatotrophin production by the anterior pituitary gland concomitantly with a depression of gonadotrophin output.

It is of interest to note, however, that the depression of oxygen consumption (an anticipated effect of lowered blood protein-bound iodine, due to injection of adrenal cortical hormones) is not consistently observed in homoiotherms; there being reported a decrease (Marine and Bauman, 1922; Franke, 1932; Hill *et al.*, 1950), increase (Koehlar and Hastings, 1929), and no change (Harrop *et al.*, 1931; Webster *et al.*, 1932; Hitchock *et al.*, 1938; Missiuro *et al.*, 1938) in resting oxygen consumption. Yet, in the cold-blooded animal, a depression of oxygen consumption is consistently observed (Calhoon and Angerer, 1950b, 1954).

It would appear, therefore, that the mechanism of the effect of adrenal cortical extract on the resting oxygen consumption of cold-blooded animals occurs via an inhibition of thyroid activity. The consistency of this effect as contrasted to that observed in warm-blooded animals, is perhaps due to the inability of the endocrine mechanisms in the poikilotherm to adjust to marked imbalances of that system.

SUMMARY

Studies were made on three groups of frogs: (a) one group of uninjected frogs exhibited a relatively constant mean oxygen consumption, (b) the second group of frogs, injected with propylthiouracil, exhibited an exponential depression of oxygen consumption, reaching a level of *ca.* 40% below the initial uninjected value, (c) the third group, injected with propylthiouracil plus adrenal cortical extract, exhibited a decrease of mean oxygen consumption so that at the end of the fifth week it was about 45% below the initial uninjected value.

It would appear that the depression of oxygen consumption observed when poikilotherms are injected with adrenal cortical extract is due to a depression of thyroid activity. This depression is due either to a direct action of the adrenal cortical hormones on the thyroid, or a suppression of thyrotrophin output from the anterior pituitary gland concomitantly with a decrease of ACTH output. Evidence is presented which suggests that the latter is the case.

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THE EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE ON S^{35} FIXATION IN CARTILAGE[1,2]

CHARLES W. DENKO[3] AND DELBERT M. BERGENSTAL

Department of Medicine and the Argonne Cancer Research Hospital, University of Chicago, Chicago, Illinois

THE studies of Dziewiatkowski *et al.* (1949), Layton (1949), Layton *et al.* (1952) and Dziewiatkowski (1952, 1953) have demonstrated that radioactive sulfur, S^{35}, is readily fixed into connective tissue, eapecially cartilage, of man, rats and mice. Cartilage is many times more active in rate of sulfur fixation than are other tissues. By isolating chondroitin sulfate $S^{35}O_4^-$, intraperitoneally, Dziewiatkowski (1951) and Bostrom (1952) showed that S^{35} is incorporated into chondroitin sulfate, the essential polysaccharide of cartilage. This sulfur uptake is a vital process since boiled cartilage shows no such activity nor is there a noteworthy uptake of S^{35} when $Na_2S^{35}O_4$ is added to pure chondroitin sulfuric acid alone (Bostrom and Mansson, 1952).

In view of the notable influence of the growth hormone of the pituitary body on the growth of cartilage (Becks *et al.*, 1949) knowledge of the effects of this principle on the metabolism of cartilage becomes essential. We have accordingly examined the uptake of injected S^{35} into the xiphoid and costal cartilages and tibial caps of normal and hypophysectomized rats both with and without stimulation by growth hormone. Assays were performed 24 hours after the last injection in a series of doses of S^{35} and growth hormone. At this time the incorporated sulfur probably lies largely in chondroitin sulfate although small amounts may be in other sulfur compounds. Since the preparations of growth hormone available to us were contaminated by thyrotrophin suitable experiments using thyrotrophin were performed as well. Some comparative data on other tissues are also noted.

While this work was in progress, Ellis *et al.* (1953) reported that hypophysectomy decreased S^{35} fixation in the costal cartilage of rats and that growth hormone administration resulted in an approximate two- or threefold increase in S^{35} fixations in the costal cartilage. Our work is in agreement

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[3] Fellow of the Arthritis and Rheumatism Foundation.

with their observations and is extended to cover the tibial caps and xiphoid processes as well as the costal cartilages. We have used a multiple dose technique for the administration of S^{35} and growth hormone providing a longer contact of S^{35} and a longer time of operation of the hormone than used in the California studies.

EXPERIMENTAL

Young female rats, hypophysectomized and normal, were used as test animals.[4] Hypophysectomy was performed at age 22 days shortly after weaning. Usually the animals were rested for three to six days before being used experimentally. A number of animals were studied 24 days after hypophysectomy. Litter-mate normal rats were used as controls. All animals weighed approximately the same at the beginning. All animals were housed under similar conditions in an air-conditioned room with controlled temperature. They were fed a mixture of milk, bread and canned dog food *ad libitum*. Dietary supplements of carrots and lettuce were given every few days. Dog food pellets were also freely available. Tap water was available for drinking.

The untreated hypophysectomized rats usually gained about five grams in weight in nine days of observation as compared to thirty-five grams in their litter-mate controls. Weights were measured to the nearest gram. At autopsy the target endocrine glands were very small as compared with the normal. Several animals were examined grossly at autopsy with no macroscopic evidence of pituitary tissue. Serial sections were not done. Growth hormone may be considered to be present in only minute amounts in hypophysectomized animals if there at all.

The growth hormone used was Armour, lot number R 491132. This contained thyrotrophin and pressor substances as impurities. Thyrotrophin (TSH) was present at the level of 0.04 ± 0.02 U.S.P. units/mg. Since growth hormone was used at the level of 100 mcg. per dose, it contained TSH as a contaminant at about 0.004 U.S.P. units per dose. The thyrotrophin used in these studies was Armour, lot number J 11002, containing no appreciable amount of impurity. Three milligrams of this material was equal to one U.S.P. unit of TSH. Therefore as a control for the growth hormone it was given at 0.004 U.S.P. units or 0.012 mg. of thyrotrophin per dose. In all experiments the controls received saline comparable in volume to that of the hormone solution.

Two attempts were made to compare the effects of active growth hormone with inactivated material. Slight inactivation from the standpoint of effects on body weight was achieved by incubating a solution of growth hormone at pH 3 at 37° C. for one hour. The pH was then adjusted to pH 8 and the resulting solution was then injected into hypophysectomized rats in the same manner as the untreated growth hormone. In another study a suspension of the growth hormone preparation previously coagulated by immersion in boiling water for twenty minutes was used.

Sulfur 35 was obtained from the Atomic Energy Commission Oak Ridge Laboratories, Oak Ridge, Tennessee, carrier-free as inorganic sulfate. The actual strength was $\pm 20\%$ of the theoretical. The S^{35} was diluted in normal saline at convenient concentrations, usually 20 microcuries per ml. Growth hormone solution was usually made to contain the S^{35} in saline, thus growth hormone and S^{35} were given simultaneously intraperitoneally at 100 mcg. and 10 microcuries respectively per dose per day for eight doses in nine days. All animals received the same dosage of S^{35} or growth hormone regardless of body weight with the exception of the experiment involving hypophysectomized animals 24 days after hypophysectomy compared to those 4 days after hypophysectomy. Higher doses of sulfur, 20 μc., were given in this latter case thus yielding higher counts

[4] Obtained from Hormone Assay, Inc., Chicago, Illinois.

than in other groups of animals. Experiments were carried out with small groups of animals usually 4 or 5 in each group. Experiments were then usually repeated two or more times. No correction is made for radio decay since comparisons are not quoted between different sets of experiments. All comparative data are obtained under similar conditions.

About 24 hours after the animals had received the last injections, they were sacrificed by ether and tissues were removed for study. The tissues were dissected clean, weighed and comparable sized samples were taken. These samples weighed 10 to 100 mg. Those that could not be processed on the day they were removed were kept in the deep-freeze. The ribs from both sides and the xiphoid were dissected free from adjacent tissues and cut so that only pure cartilage was obtained. The tibial cap was removed uniformly at the metaphyseal-epiphyseal junction. Usually two or more samples of the cartilages were taken from each animal. Other tissues, e.g., lymph node and skeletal muscle, were dissected free at the same time as the cartilage and processed wet in a simliar manner. Muscle, cardiac and skeletal, was found to be low in activity. This low activity was determined repeatedly to exclude contamination of tissues or equipment by extraneous S^{35}.

Tissues were ashed with anhydrous Na_2CO_3 in a platinum crucible. The resulting ash was dissolved in normal HCl and the resulting solution was adjusted to the acid pH of phenol red indicator. This solution in a 100 ml. beaker was heated on a hot plate and the sulfate was precipitated by adding as carrier 5 ml. of 0.1 N Na_2SO_4 and 3 ml. of N $BaCl_2$. The suspension remained covered and was heated on the hot plate below the boiling point for at least four hours. Then it was cooled and afterwards filtered using a Tracerlab metal filter. The $BaSO_4$ on the filter pad (Whatman No. 240; 2.4 cm. diameter) was washed with water and dried with ethanol. The precipitate weighed approximately 65 mg. per paper or 25 mg. per cm^2. Counting was done with a windowless gas flow counter using helium or Q gas. Suitably high levels of activity in samples were sought so counting could be done for five minutes per sample with a 3–5% probable error in counting.

RESULTS AND DISCUSSION

Comparable samples, e.g., the right and left tibial caps, could be removed and treated so that the results would check fairly accurately with each other (Table 1). Approximately 90% of the samples of tibial caps so matched checked within about 10% each other's values. The costal cartilages exhibited somewhat less precision in regards to reliability and sampling in that approximately 80% of the matched samples of costal cartilage from opposite sides of the body checked within about 20% of each other's values. The spread of all values is indicated in Figure 1.

The cartilages of the hypophysectomized rat retained a substantial capacity to fix injected S^{35}. Indeed the tibial caps of hypophysectomized rats retained as much radioactive sulfur as did those of the normal when expressed as counts of S^{35} per minute per tibial cap (Table 1). When the fixation is expressed as counts per minute per 100 mg. of wet tissue the tibial caps of the hypophysectomized animals may even appear to exceed the normal slightly (Fig. 1). The xiphoid cartilage of the hypophysectomized rat fixed as much radioactive sulfur per 100 mg. of wet tissue as did that of the normal (Table 2, Fig. 1). Only in the case of the costal cartilage does hypophysectomy greatly reduce the uptake of radioactive sulfur.

TABLE 1. RELIABILITY OF SAMPLING (AS ILLUSTRATED BY THE WEIGHTS AND COUNTS OF THE RIGHT AND LEFT TIBIAL CAPS FROM REPRESENTATIVE ANIMALS)*

Group	Weight*		Tibial cap	
	Body weight (g.)	Tibial caps (mg.)	cpm.† of S^{35}	cpm./100 mg.
H	59	38.3	745	1900
		37.0	705	1900
	58	36.4	800	2180
		33.3	600	1770
H+GH	70	42.7	2020	4740
		41.2	1965	4780
	73	37.0	1260	3420
		38.2	1340	3520
N	110	54.5	760	1390
		54.2	725	1340
	107	60.2	950	1600
		56.7	900	1600
N+GH	119	67.0	830	1250
		68.5	960	1400
	119	57.7	740	1300
		58.2	720	1250

H = hypophysectomized rat receiving S^{35}.
H+GH = hypophysectomized rat receiving S^{35} plus growth hormone.
N = normal rat receiving S^{35}.
N+GH = normal rat receiving S^{35} plus growth hormone.
* Weight of individual animals at end of experiment.
† cpm. = counts per minute.

It is unlikely that hormones still circulating four to thirteen days after the removal of the hypophysis were responsible for the high rate of fixation of S^{35} in our animals. A small group receiving S^{35} twenty-four days after hypophysectomy did not fix less sulfur than a group receiving S^{35} four days after hypophysectomy (Table 3).

It may be considered possible that hypophysectomies were in some slight degree incomplete. The weight gain of our hypophysectomized animals during the 9 days of the experimental period averaged 5 gm. as compared to 35 gm. for their normal controls. If residual hormone is responsible for this slight weight gain and likewise influences the uptake of radioactive sulfur, the cartilages must be sensitive indeed to hypophyseal agents since the S^{35} uptake of the cartilage of the operated animals approaches the normal much more closely than does their gain in body weight.

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE ON CONNECTIVE TISSUE OF THE RAT

Tissue	Average S^{35} count per minute per 100 mg.							
	Group 1				Group 2			
	*H	H+GH	N	N+GH	H	H+GH	N	N+GH
Costal cartilage	(8) 1000 ±365	(8) 3275 ±680	(6) 1910 ±480	(6) 1930 ±370	(10) 1080 ±220	(15) 4550 ±1140	(11) 3305 ±495	(17) 2600 ±460
Tibial cap	(8) 1500 ±240	(8) 2580 ±515	(6) 860 ±110	(6) 920 ±220	(8) 1930 ±290	(9) 3730 ±770	(6) 1260 ±285	(12) 1325 ±300
Xiphoid cartilage	(3) 1970 ±380	(4) 4170 ±1400	(3) 1420 ±165	(3) 1270 ±125	(4) 3065 ±925	(5) 4840 ±1235	(4) 2450 ±455	(6) 2335 ±1090
Meniscus	(8) 685 ±260	(6) 1380 ±610	(4) 465 ±90	(5) 455 ±160	—	—	—	—
Ear	—	—	—	—	(1) 390	(3) 510	(2) 260	(3) 260
Eye coats	—	—	—	—	(1) 530	(4) 815	(3) 360	(1) 210
Lymph node	(4) 180	(4) 230	(2) 90	(2) 55	(3) 195	(1) 185	(1) 255	(2) 0
Pituitary	—	—	(2) 190	(2) 175	—	—	—	—
Ovary	—	—	(2) 95	(2) 125	—	—	—	—
Skeletal muscle	(1) 50	(1) 80	(1) 50	(1) 15	—	(1) 75	(1) 50	(1) 25
Cardiac muscle	—	—	—	—	(1) 100	(1) 140	(1) 40	(1) 30

* H = hypophysectomized rats.
H+GH = hypophysectomized + growth hormone.
N = normal rats.
N+GH = normal + growth hormone.
Figures in each column represent as follows:
1) figure in parentheses = number of samples.
2) average.
3) standard deviation of the mean, or standard error.
Probability values—p. 65; "Statistical Methods," Snedecor—4th ed. Iowa State College Press.
Group 1. costals—H vs. H+GH−P = <0.01; H vs. N−P<0.01
tibial cap—H vs. H+GH−P = <0.01
xiphoid—H vs. H+GH−P=0.3
meniscus—H vs. H+GH−P = <0.05
Group 2. costals—H vs. H+GH−P = <0.01; N vs. N+GH−P = <0.01; H vs. N−P = <0.01
tibial cap—H vs. H+GH−P = <0.01
xiphoids—H vs. H+GH−P = <0.05

It is more probable that the persistence of substantial activity in the cartilages of the hypophysectomized animals lies in the vital processes of this particular tissue which continue even though growth is in abeyance.

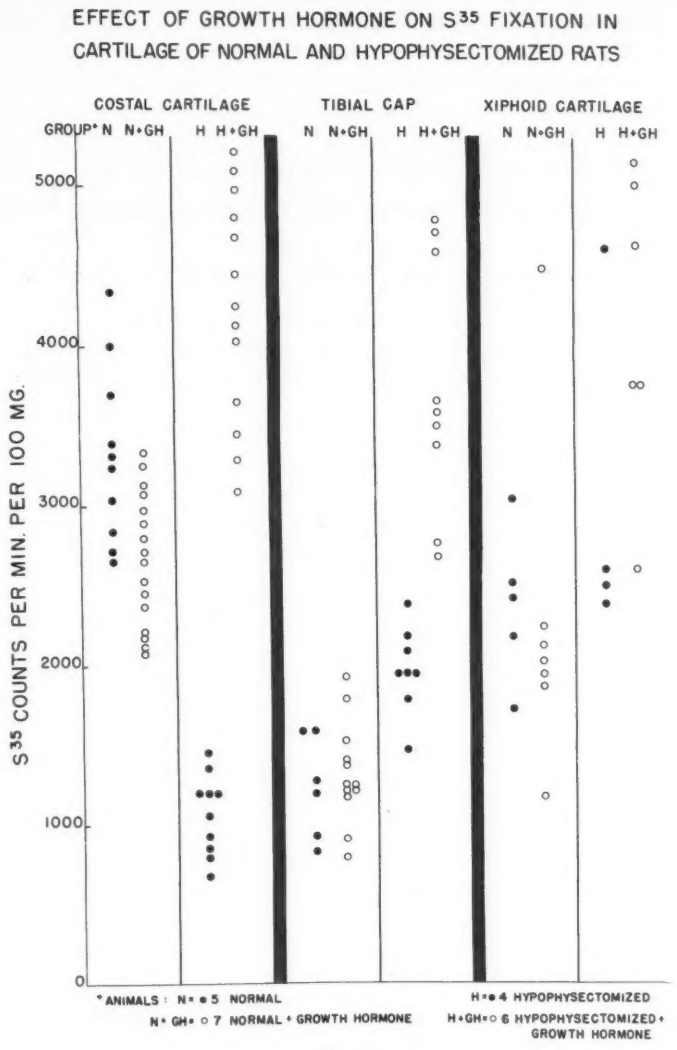

FIG. 1

TABLE 3. S^{35} FIXATION IN CARTILAGE OF HYPOPHYSECTOMIZED RATS AGED 46 AND 26 DAYS

Tissue	Counts per minute per 100 mg	
	*46 days	†26 days
Costal cartilage	7125 ± 1430	3330 ± 675
Tibial cap	5060 ± 955	5110 ± 375
Xiphoid cartilage	10,025 ± 1350	9800 ± 495

* Average of 4 animals with standard deviation of the mean or standard error.
† Average of 3 animals with standard deviation of the mean or standard error.

These vital activities may well be independent of hormonal influences. They are presumably enzymatic since boiled cartilage does not fix S^{35}. Transsulfatation proceeding in tissues containing sulfated compounds, e.g., chondroitin sulfate, may well be involved.

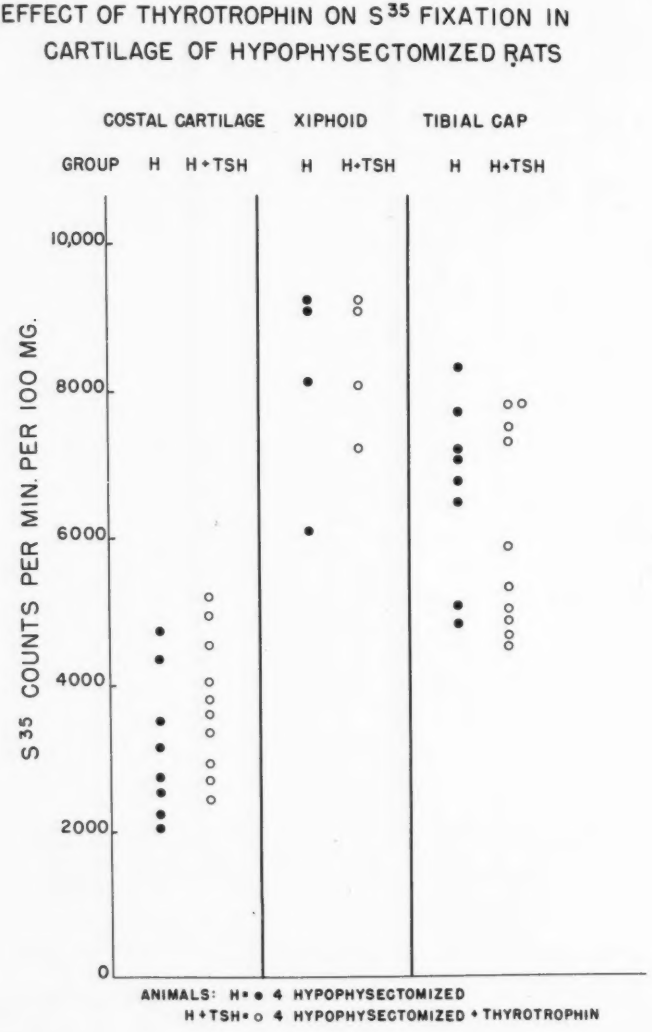

FIG. 2

Despite the circumstance that hypophysectomy has a limited influence on the uptake of S^{35} by cartilage, really distinct only in the case of costal cartilage as noted above, growth hormone stimulated fixation of S^{35} by all

three cartilages (Table 2, Fig. 1). One hundred micrograms daily of the preparation used enhanced S^{35} uptake by costal cartilage 3- to 4-fold in 9 days. The effect on the tibial cap was somewhat less but still striking. The effect on the xiphoid is less impressive perhaps because of the spread in the control data. In general the values for S^{35} fixation were carried to hypernormal levels. This was especially true of the tibial cap. The increment in body weight during the 9 days of these studies averaged 12 gm. for treated rats as compared to 5 gm. for the hypophysectomized control. The enhanced uptake of radioactive sulfur is a more dramatic expression of the influence of growth hormone than the increment in body weight.

S^{35} FIXATION AND WEIGHT INCREASE IN HYPOPHYSECTOMIZED RATS RECEIVING GROWTH HORMONE, INACTIVATED, AND PARTIALLY INACTIVATED GROWTH HORMONE

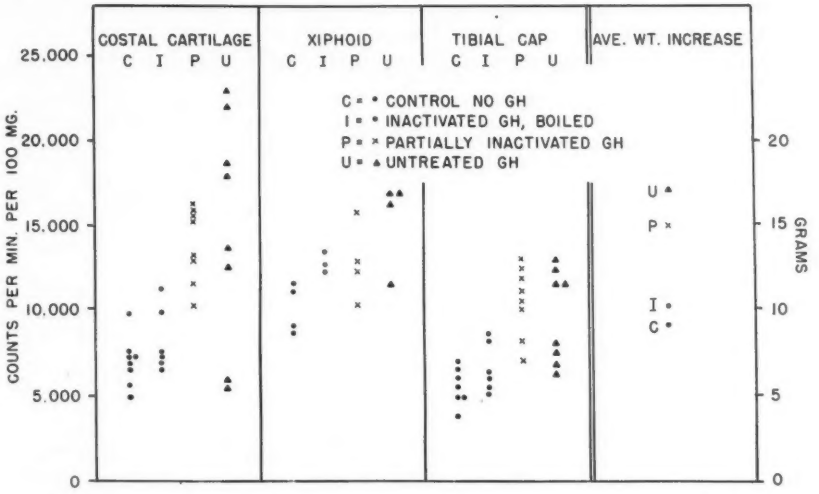

FIG. 3

The influence of our growth hormone preparation was not due to contaminating thyrotrophin. Purified thyrotrophin injected for eight doses in nine days at 0.004 U.S.P. units per dose, the amount present as a contaminant in our growth hormone preparation, caused no notable effect on S^{35} fixation in any of the cartilages studied (Fig. 2). This does not exclude the possibility that the traces of thyrotrophin in our growth hormone preparations might have served synergistically with the growth hormone itself. It does show, however, that thyrotrophin alone could not have been responsible for the increased uptake of radioactive sulfur.

Attempts to inactivate the growth hormone and to test the effects of the inactivated material were made. Complete inactivation (see Experimental) eliminated all influence on S^{35} fixation and on weight increase (Fig. 3).

Heating at 37° C. at pH 3 for one hour (see Experimental) reduced slightly the capacity of this material to stimulate the uptake of radioactive sulfur in costal cartilage and to increase body weight.

It is therefore quite likely that the growth hormone itself is the hypophyseal factor enhancing the uptake of S^{35} by cartilage and that this enhancement bears some relation to the typical chondrotrophic or growth promoting activity of this agent. Our data, however, do not permit a statement as to whether the uptake of S^{35} in a given mass of reacting sulfate was increased as by enhanced transsulfatation or whether the actual mass of reacting sulfate incorporating S^{35} was increased by growth hormone or whether both processes were affected. Nor do they in respect to any process distinguish between enhanced synthesis and retarded degradation.

It is of interest that this influence of the growth hormone was exhibited

TABLE 4. EFFECT OF GROWTH HORMONE ON S^{35} FIXATION IN CARTILAGE OF AN EXCEPTIONAL ANIMAL WHICH DID NOT GAIN WEIGHT

Tissue	H	H+GH
	cpm./100 mg.	
Costal cartilage	650	3200
Tibial cap	1150	2600
Xiphoid cartilage	1550	5840
Weight gain*	g.	
	5	1

* One animal in each group.
H = hypophysectomized rat.
H+GH = hypophysectomized plus growth hormone.

by a few animals in which weight gain did not occur for some unknown reason. Thus, factors inimical to the response of the soma generally need not prevent the responses of cartilage to the growth hormone (Table 4).

When growth hormone is present in physiological amounts as in the normal animal exogenous growth hormone appears not to increase the uptake of radioactive sulfur (Fig. 1). Indeed with respect to the costal cartilage and possibly with respect to the xiphoid an actual reduction in S^{35} uptake is suggested. The series is too small for certainty on this point. One effect, however, is quite clear. Growth hormone within the experimental time elevates S^{35} uptake in the hypophysectomized rat not only above that of the normal animal but also above that of the normal animal given the same amount of growth hormone. Further work must be undertaken to determine whether or not this is a true example of the hypersensitivity of the hypophysectomized animal to growth hormone and whether such hypersensitivity, if it exists, resides in the cartilage itself or is of more general origin.

Other connective tissues, e.g., the meniscus, behave similarly to the costal cartilage in responding to growth hormone (Table 2). However, the level of sulfur fixation in most other tissues, i.e., the ear, eye coats, lymph node, pituitary gland, ovary, and muscle, is too low in comparison to cartilage to be utilized satisfactorily in this study.

SUMMARY

Hypophysectomy of rats at age 23 days results in a decrease by $\frac{1}{3}$ to $\frac{1}{2}$ in the S^{35} fixation in the costal cartilage when compared to the costal cartilage of young normal rats. Tibial caps and xiphoid cartilage do not exhibit this marked reduction. The ability of the costal cartilage of the hypophysectomized rat to fix sulfur is present in animals hypophysectomized 24 days before the administration of labeled sodium sulfate.

Growth hormone administered to the hypophysectomized rat results in increased fixation of S^{35} in the costal, xiphoid, and tibial cap cartilages. The S^{35} uptake by costal cartilage was increased three- to four-fold by administration of 100 micrograms daily of growth hormone for eight days. In the hypophysectomized rat the S^{35} fixation following growth hormone is higher than that seen in the normal rat or in the normal rat given growth hormone in the doses we have used.

When growth hormone is given to a normal rat at doses comparable to those producing stimulation of S^{35} fixation in the hypophysectomized rat to levels well above the normal rat it does not stimulate fixation of S^{35}. In fact, an actual reduction in S^{35} fixation is suggested.

Purified thyrotrophin injected in the amount present as a contaminant in our growth hormone preparations caused no notable effect on S^{35} fixation in any of the cartilages studied.

Factors inimical to weight gain in rats do not prevent the response of cartilage to growth hormone.

Heating of growth hormone at 37° C. at pH 3 for one hour decreases slightly its ability to stimulate S^{35} fixation in costal cartilage and to increase weight, while complete inactivation of growth hormone abolishes the stimulation of S^{35} fixation and weight increase.

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COMPARATIVE STUDIES ON HYDROLYSIS OF CONJUGATED ESTROGENS IN HUMAN PREGNANCY URINE

RICHARD F. STRAW,[1] PHILIP A. KATZMAN AND EDWARD A. DOISY

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri

WE HAVE previously reported (Katzman *et al.*, 1954) comparative studies on the hydrolysis of the conjugated estrogens of human pregnancy urine by bacterial β-glucuronidase, the phenolsulfatase of Mylase P, and boiling 15 vol.% HCl. In most instances, enzymatic hydrolysis liberated somewhat more estrogen than was obtained by treatment with acid. Use of the enzymes also permitted study of the nature of the conjugated estrogens. β-Glucuronidase obtained from culture filtrates of *E. coli* liberated most of the estrogen while only a small amount was liberated by the phenolsulfatase of Mylase P. Moreover, certain observations indicated that Mylase P liberates more estrogen from urines previously hydrolyzed with β-glucuronidase than from the same urines not so treated.

One aspect of this paper is an extension of our previous work on the amount of estrogen liberated by phenolsulfatase prior to and subsequent to hydrolysis with β-glucuronidase. The total amount obtained by hydrolysis with both enzymes used simultaneously has been compared with that found by hydrolysis with zinc and hydrochloric acid. Since we had found somewhat more estrogen by enzymatic than by acidic hydrolysis, it seemed advisable to use hydrochloric acid and zinc because Smith and Smith (1941) have reported that the addition of zinc leads to higher values. This observation has been confirmed by Stimmel (1949), Heard and Saffron (1949), and Stimmel, Grollman and Huffman (1950). Some investigators (Venning, Evelyn, Harkness, and Browne, 1939; Stimmel, 1946) have employed aqueous solutions of the butanol extractable substances of urine, rather than whole urine for hydrolysis and estimation of estrogens. In the present report, we have extended our studies to include this procedure.

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[1] The material presented herein was taken from a dissertation submitted to the Graduate School of St. Louis University by Richard F. Straw in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry. Present address: Department of Pathology, St. Francis Hospital, Wichita 5, Kansas.

In general, we have found by colorimetric analysis that 1) hydrolysis of urines with 15 vol.% HCl in the presence of zinc yielded somewhat more Kober chromogenic material than HCl alone; 2) hydrolysis of aqueous solutions of the butanol extractable substances of urines yielded amounts of estrogen as high as or slightly higher than those obtained by the same treatment of the whole urines; 3) the amount of estrogen liberated by simultaneous hydrolysis with β-glucuronidase and Mylase P was approximately the same as liberated by these enzymes employed separately; and 4) experimental evidence was obtained which indicates that a small amount of estriol is excreted as a double conjugate with sulfuric and glucuronic acids.

EXPERIMENTAL

The methods employed in this study unless otherwise specified were the same as those described previously (Katzman, *et al.*, 1954). Urines A through F are the same urines which were used in that publication. Urine H was a sample of pooled 24-hour urine specimens. All were extracted with ether to remove free estrogens before the hydrolyses were undertaken. Estrogen was estimated by a modified Kober method and interfering material corrected for by means of a nomogram (Katzman, *et al.*, 1954).

Hydrolysis with 15 vol.% HCl in the presence of zinc. Aliquots of the urines were heated to boiling in the presence of 4 gm.% zinc dust and then

TABLE 1. A COMPARISON OF THE AMOUNTS OF ESTROGENS FOUND IN URINE AFTER HYDROLYSIS BY ENZYMES AND BY 15 VOLUMES PER CENT HCl WITH AND WITHOUT ZINC

Urine	Method of hydrolysis				
	15 vol. % HCl, 4% Zn			15 vol. % HCl 30 min.	Enzymatic simultaneous MP+G
	2 hrs.	3 hrs.	4 hrs.		
	Total estrogen expressed as mg. estriol/24 hrs.				
A	14.9	15.3	—	13.4	16.3
B	10.6	11.3	10.2	9.5	13.3
C	10.3	12.2	11.6	11.5	12.0
D	13.9	13.9	15.0	12.1	14.5
E	19.1	19.7	24.0	18.2	20.0
F	8.5	9.3	12.6	12.2	11.6

MP—Mylase P.
G—Bacterial β-glucuronidase.

sufficient concentrated HCl was added to make a final concentration of 15 vol.%. The results (Table 1) show that maximum values were obtained by 3 or 4 hours of refluxing. Longer heating, up to 9 hours in some cases, gave no further increase. Comparison of the values obtained by this method with those obtained by other methods (last two columns) indicates that this method of hydrolysis gave somewhat higher values than those obtained with HCl alone, but that there was no appreciable increase, as determined by the Kober method, over the values obtained by enzymatic hydrolysis, except in the case of urine E. Partition of the estrogens obtained by Zn-HCl

treatment into estrone-estradiol and estriol fractions indicated that the increase observed occurred in both fractions.

These increases (0–32%) for hydrolysis with acid in the presence of zinc are not as great as those reported by Stimmel (1949). Part of the difference between our results and those of Stimmel (1949) may be accounted for by the fact that Stimmel subjected the urines to only 10 minutes of refluxing with HCl, which we found to give from 10 to 42% less estrogen than that obtained by 30 minutes of refluxing (Katzman, *et al.*, 1954).

Enzymatic hydrolysis. From 100 to 200 units (Talalay, Fishman, and Huggins, 1946) of β-glucuronidase were added for each ml. of pregnancy urine; Versene was added to give a concentration of 100 mg.% and the pH

TABLE 2. TOTAL FREE ESTROGENS AFTER HYDROLYSIS WITH MYLASE P (MP) AND β-GLUCURONIDASE (G) USED IN DIFFERENT SEQUENCE AND SIMULTANEOUSLY

Urine	Sequence of treatment		
	MP-G	G-MP	MP and G simultaneously
	Total estrogen expressed as mg. estriol/24 hrs.		
A	15.5	15.7	16.3
B	11.9	12.6	13.3
C	11.5	13.0	12.0
D	14.2	15.3	14.5
E	19.6	21.9	20.0
F	12.1	12.6	11.6
H	20.3	20.0	20.6

adjusted to 6.5–7.0. Incubation was conducted at 37° C. for 24 hours. Mylase P was used at a concentration of 1% and the urine incubated at pH 6.0–6.5 at 37° C. for 24 hours. The estrogens liberated by incubation with each enzyme were exhaustively extracted with ether. The ether extracts were washed once with 1/10 vol. H_2O and this washing added to the urine, which was then heated on a boiling water bath to remove ether and destroy the enzyme. The other enzyme was then used. The estrogens liberated by each enzyme were determined separately and the total estrogen obtained by adding these values.

When both enzymes were employed simultaneously, they were used at the levels indicated above. Versene was added to a concentration of 100 mg.%, the pH was adjusted to 6.0–6.5 and the samples incubated at 37° C. for 24 hours. In all cases, a small amount of chloroform was added as a preservative.

The results, shown in Table 2, indicate that the total estrogen obtained by simultaneous incubation with both enzymes compares favorably with that obtained by the use of the enzymes separately in either sequence.

In our previous studies (Katzman, *et al.*, 1954), it was noted that more estrogen was recovered by Mylase P treatment from urines which had been incubated with β-glucuronidase than from untreated urines. A series

of experiments was performed to ascertain if this increase may have been due to incomplete extraction after the glucuronidase treatment or to continued action by β-glucuronidase during the treatment with Mylase P. Urines were hydrolyzed with β-glucuronidase and the estrogens extracted. These urines were then subjected to a second treatment with β-glucuronidase after which the usual series of ether extractions was performed twice. This procedure yielded very small quantities of additional estrogen which did not account for the increase observed in our earlier work (Katzman, *et al.*, 1954). Treatment of these urines with Mylase P liberated more estrogen than was obtained by Mylase P from samples of the same urines before glucuronidase treatment.

TABLE 3. COMPARISON OF THE AMOUNTS OF ESTROGEN LIBERATED FROM PREGNANCY URINE BY MYLASE P (MP) AND β-GLUCURONIDASE (G) SEPARATELY IN DIFFERENT SEQUENCE

Urine	Amount of estrogen liberated by enzymatic hydrolysis			
	Mylase P		β-Glucuronidase	
	Before G	After G	Before MP	After MP
	Expressed as mg. estrone/24 hrs.*		Expressed as mg. estriol/24 hrs.	
A	0.7 (3)	1.6 (2)	14.1 (3)	13.1 (2)
B	0.6 (3)	1.6 (2)	11.0 (2)	11.3 (3)
C	0.7 (4)	2.0 (3)	11.0 (2)	10.8 (3)
D	0.8 (4)	1.5 (3)	13.8 (3)	13.4 (3)
E	0.5 (2)	1.8 (2)	20.1 (2)	19.1
F	0.5	1.1	11.5	11.6
H	0.7 (2)	2.0 (2)	18.0 (2)	19.6 (2)
R	0.4 (4)	0.8 (3)	11.6 (3)	12.0 (2)
X	1.2 (2)	1.8 (2)	24.3 (2)	25.5 (2)
Y	0.8 (2)	1.9 (2)	22.3 (2)	24.8 (2)

Numbers in parenthesis indicate the number of complete experiments performed.
* Since estrone is more chromogenic than estriol toward Kober's reagent and since the estrogen of these fractions is predominantly estriol, these values would be approximately 20% higher were they expressed as estriol instead of estrone.

Samples of ten urines were then subjected to hydrolysis by the enzymes in each sequence. To insure the maintenance of an adequate concentration of glucuronidase, 50% more glucuronidase was added midway through the incubation period. Following this treatment, the usual series of ether extractions was performed twice before treatment with Mylase P. The results of these experiments are shown in the first two columns of Table 3. In every instance the amount of estrogen liberated by Mylase P was greater after hydrolysis with β-glucuronidase than before. While the absolute amounts of estrogen involved are relatively small, the percentage differences (50–250%) are far greater than can be attributed to errors of assay.

If the above results are due to the presence of estrogen conjugated with both sulfuric and glucuronic acids, then hydrolysis with β-glucuronidase should yield more estrogen after Mylase P treatment than before. Since

the differences in the absolute values are usually within the experimental error of the Kober analyses, the data in the last two columns of Table 3 do not support this. In the case of the figures given under Mylase P, the absolute differences are of the same order of magnitude but the percentage differences are far beyond the error of the analytical method and consequently are significant.

To obtain further information on the estrogens liberated by Mylase P after previous hydrolysis with β-glucuronidase, two 2-liter samples of urine H (see Table 3), one having previously been treated twice with glucuronidase and the liberated estrogen removed by a series of extractions with ether after each treatment, were hydrolyzed with Mylase P. The estrogens obtained were separated into estriol and estrone-estradiol fractions. The results, Table 4, indicate that the additional estrogen appeared in the estriol fraction.

TABLE 4. FRACTIONATION OF THE ESTROGENS LIBERATED BY MYLASE P FROM PREGNANCY URINE BEFORE AND AFTER TREATMENT WITH β-GLUCURONIDASE

	Estrogen liberated by Mylase P		
	Total	Estriol	Estrone-estradiol
	Expressed as mgs. of estrone*		
Before β-glucuronidase	0.7	0.5	0.2
After β-glucuronidase	2.0	1.7	0.2

* If estriol had been used as the standard, the absolute values for total estrogen and for estriol would have been somewhat larger. However, the relative values clearly show the difference discussed in the text.

The estriol fraction liberated by Mylase P after glucuronidase was subjected to countercurrent distribution by the methods described by Engel (1950) in the 25 tube stainless steel apparatus described by Craig and Post (1949).

The fraction was first distributed in the 50% aqueous methanol/carbon tetrachloride solvent system. After removal of aliquots for Kober determination, the contents of tubes 20–24 were pooled and distributed in the 50% cyclohexane-50% ethyl acetate/50% aqueous ethanol solvent system. The results are shown graphically in Figure 1. The values of K ($0.24 \pm .04$) calculated for the material at the peak in the solvent system 50% cyclohexane-50% ethyl acetate/50% aqueous ethanol is in good agreement with that ($K = .29 \pm .03$) which we obtained for a sample of pure estriol under the same conditions. However, these values are not in close agreement with that ($0.40 \pm .08$) reported by Slaunwhite, *et al.* (1951). The lack of agreement between the coefficients for estriol as determined by Slaunwhite *et al.* (1951) and by ourselves may reflect only differences in preparation of the solvents. However, in view of the value for K obtained in our control work on the authentic estriol in which the same batches of

solvents were used, we conclude that estriol accounts for the increased amount of estrogen liberated by Mylase P after glucuronidase.

Hydrolysis of conjugated estrogens in butanol extracts of urine. Butanol extracts were prepared as follows: aliquots of urine were acidified to pH 3 and extracted 3 times with $\frac{1}{3}$ volume of butanol. The combined extracts were washed once with 1/10 volume of water, and the washing was discarded. In order to effect distillation at approximate neutrality, the butanol extracts were neutralized by washing with small quantities of 5% $NaHCO_3$ until the washings remained alkaline. These washings were then

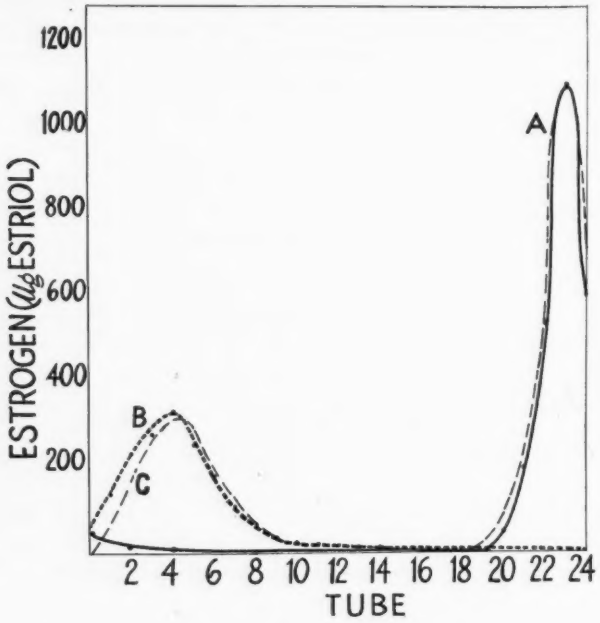

FIG. 1

neutralized, combined with the butanol and the mixture distilled to dryness under reduced pressure at 50–60° C. The residues were taken up in water, the pH adjusted to 7 and the volumes adjusted to the original volumes of the aliquots.

These aqueous solutions of the butanol extractable substances of urine were then subjected to the various methods of hydrolysis. The results are shown in Table 5 which include for comparison the values for total estrogen obtained from the whole urines by similar treatment. The last figure in each column is the average ratio of absorption at 540 mμ to absorption at 420 mμ observed in the Kober determination, and is presented as an indication of the relative amount of interfering material in the final estrogen fraction.

NOTICE OF OMISSION

In the article by Richard F. Straw, Philip A. Katzman and Edward A. Doisy, ENDOCRINOLOGY 57:87. 1955, the legend to Figure 1 was inadvertently omitted. It is given below:

Fig. 1. Countercurrent distribution of the estriol fraction liberated by Mylase P after β-glucuronidase. Estriol was obtained from the total estrogenic fraction by the procedure (Katzman, *et. al.*, 1954) in use in this laboratory. Curve A: Estriol fraction from urine in 50% aqueous methanol: CCl_4. Curve B: Estriol fraction from urine in 50% cyclohexane-50% ethyl acetate: 50% aqueous ethanol. Curve C: Theoretical curve for authentic estriol.

The data indicate that hydrolysis with enzymes or 15 vol.% HCl yielded somewhat more estrogen from butanol extracts than from whole urines in all instances and significantly more in some. This was not observed when 15 vol.% HCl was used in the presence of zinc. It may be noteworthy that although HCl in the presence of zinc yielded higher values than those obtained with HCl alone if whole urines were used, this difference was not observed when butanol extracts were employed. It would appear from the ratios of the absorption at 540 mμ to that at 420 mμ that the amount of interfering material in the estrogen fractions obtained by acid hydrolysis is the same whether butanol extracts or whole urines were used. More interfering material was present in the fractions obtained by enzymatic hydrolysis of butanol extracts than in those obtained from whole urines.

TABLE 5. A COMPARISON OF THE AMOUNTS OF ESTROGEN LIBERATED FROM PREGNANCY URINES AND THEIR BUTANOL EXTRACTS BY DIFFERENT METHODS OF HYDROLYSIS

Urine	Method of hydrolysis							
	15 vol. % HCl		15 vol. % HCl, 4% Zn		β-glucuronidase		Mylase P and glucuronidase simultaneous	
	Whole urine	Butanol extract	Whole urine	Butanol extract	Whole urine	Butanol extract	Whole urine	Butanol extract
	Expressed as mg. estriol/24 hrs.							
A	13.4	15.2	15.3	13.4	14.2	15.2	16.3	17.7
B	9.5	11.6	11.3	11.0	10.2	12.7	13.3	14.4
C	11.5	12.4	12.2	12.0	10.4	10.8	12.0	14.0
D	12.1	15.8	15.0	14.5	13.8	13.8	14.5	14.5
E	18.2	23.0	24.0	21.8	19.6	22.4	20.0	23.5
540/420 ave.	2.2	2.2	2.7	2.9	4.3	2.7	3.3	2.9

DISCUSSION

The observation that preliminary treatment of human pregnancy urine with β-glucuronidase increased the estrogen liberated by Mylase P suggests that some estrogen is excreted as a double conjugate. The fact that the additional estrogen appeared in the estriol fraction and behaved like estriol upon countercurrent distribution in two solvent systems supports this suggestion. Our procedure appears to exclude the possibility that this increase is due to the incomplete extraction of estriol following glucuronidase hydrolysis, or the incomplete hydrolysis of estriol glucuronide during the two 24-hour incubation periods. An alternative explanation, that β-glucuronidase removes or inactivates some substance inhibitory to the phenolsulfatase of Mylase P, seems to be ruled out by our previous observations that the estrone of sodium estrone sulfate added to male's urine was quantitatively recovered by hydrolysis with Mylase P, and that the amount of estrogen obtained by initial Mylase P hydrolysis of pregnancy

urine is in good agreement with that obtained by continuous ether extraction at pH 0.7 (Katzman, *et al.*, 1954).

Smith and Williams (1949) have reported instances of double conjugation. They observed that aniline, fed to rabbits, was converted to o- and p-aminophenol and that part of the p-aminophenol was excreted as p-acetamidophenylglucuronide. They reported similar findings for p-phenetidine which was converted to p-aminophenol and excreted as p-acetamidophenylglucuronide (Smith and Williams, 1949).

From a comparison of the results obtained by the various methods of hydrolysis of conjugated estrogens in human pregnancy urine, it would seem that the method of choice is largely dependent upon the objectives of the investigator and also perhaps upon the method of assay used. While the values for the total estrogen of pregnancy urine as determined by the Kober method following acid hydrolysis do not differ greatly from those obtained after enzymatic hydrolysis, the possibility of chemical alteration is not excluded (Katzman, *et al.*, 1954). We agree with Smith and Smith (1941) and Stimmel (1949) that Zn-HCl hydrolysis is not suitable for the study of the natural pattern of estrogens in urine. For the latter objective the enzymatic methods yield comparable Kober values with less likelihood of chemical alteration of the estrogens.

The preparation of butanol extracts appears to be a valid procedure and yields slightly higher values for both acidic and enzymatic hydrolysis. In carefully controlled studies, the time and trouble involved in the preparation of butanol extracts may be worthwhile.

SUMMARY

A method is presented for the simultaneous use of Mylase P and β-glucuronidase for the hydrolysis of conjugated estrogens in pregnancy urine. The method yields values which compare favorably with those obtained by use of the enzymes separately in either sequence.

Evidence is presented which indicates that a small amount of estriol is excreted as a double conjugate with sulfuric and glucuronic acids.

Previous comparative studies on hydrolysis of conjugated estrogens in pregnancy urine have been extended to include hydrolysis with HCl in the presence of zinc. This method yields somewhat higher values than are obtained with HCl alone, but not appreciably higher than obtained by enzymatic hydrolysis.

A comparison of the principal methods of hydrolysis using whole urines and their butanol extracts indicated that somewhat higher values are obtained with the butanol extracts by enzymatic and HCl hydrolysis, but not by Zn-HCl.

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STAINING OF THE ADENOHYPOPHYSIS WITH ACID AND BASIC DYES[1]

ROY R. PETERSON AND JULES WEISS[2]

Department of Anatomy, Washington University School of Medicine, Saint Louis, Missouri

IN THE seventy years since Flesch (1884) and Dostoiewsky (1886) first distinguished chromophobes from chromophiles, the application of different cytological techniques under a variety of experimental conditions had led to an increasingly complex separation of the cell types of the adenohypophysis. The first clear division of the chromophiles was by von Schönemann (1892), who distinguished between eosinophiles, which stained with eosin, and cyanophiles, which stained with hematoxylin. It was Thom (1901) who substituted basophile for cyanophile, thereby establishing the terminology which has been employed by the majority of investigators of pituitary cytology. Thus there has been recognized a main division of pituitary cells into chromophobes, and the two types of chromophiles, namely acidophiles and basophiles. In recent years this classification has been criticised as being inaccurate because the cells are amphoteric, and, more important, because the basophiles are usually identified by staining with the acid dye anilin blue (Bailey, 1932; Romeis, 1940; Greep, 1954). One of the main difficulties in the classification of pituitary cell types on a morphological basis has centered about the problem of whether or not the cells called acidophiles and basophiles are truly acidophilic and basophilic, respectively.

The use of acid and basic dyes in staining solutions of constant dye concentration, buffer concentration, staining time, and temperature, but of varying pH, has been shown by Singer (1952) and Weiss (1953) to be a valuable tool in helping to characterize the relative acidity or basicity of tissue proteins, and other insoluble electrolytes. The controlled pH staining method has been utilized in the present study to determine these characteristics for the different classes of pituitary cells in the cat, rabbit, guinea pig, rat and mouse. It was found that the granules of the acidophile cells were indeed relatively acidophilic and that the pH staining curves were strikingly similar in all species. The basophile cell granules were rela-

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tively basophilic, but the pH staining curves differed in some species. In addition previous reports on the localization and distribution of the Golgi-containing macula and the ergastoplasm were confirmed.

MATERIALS AND METHODS

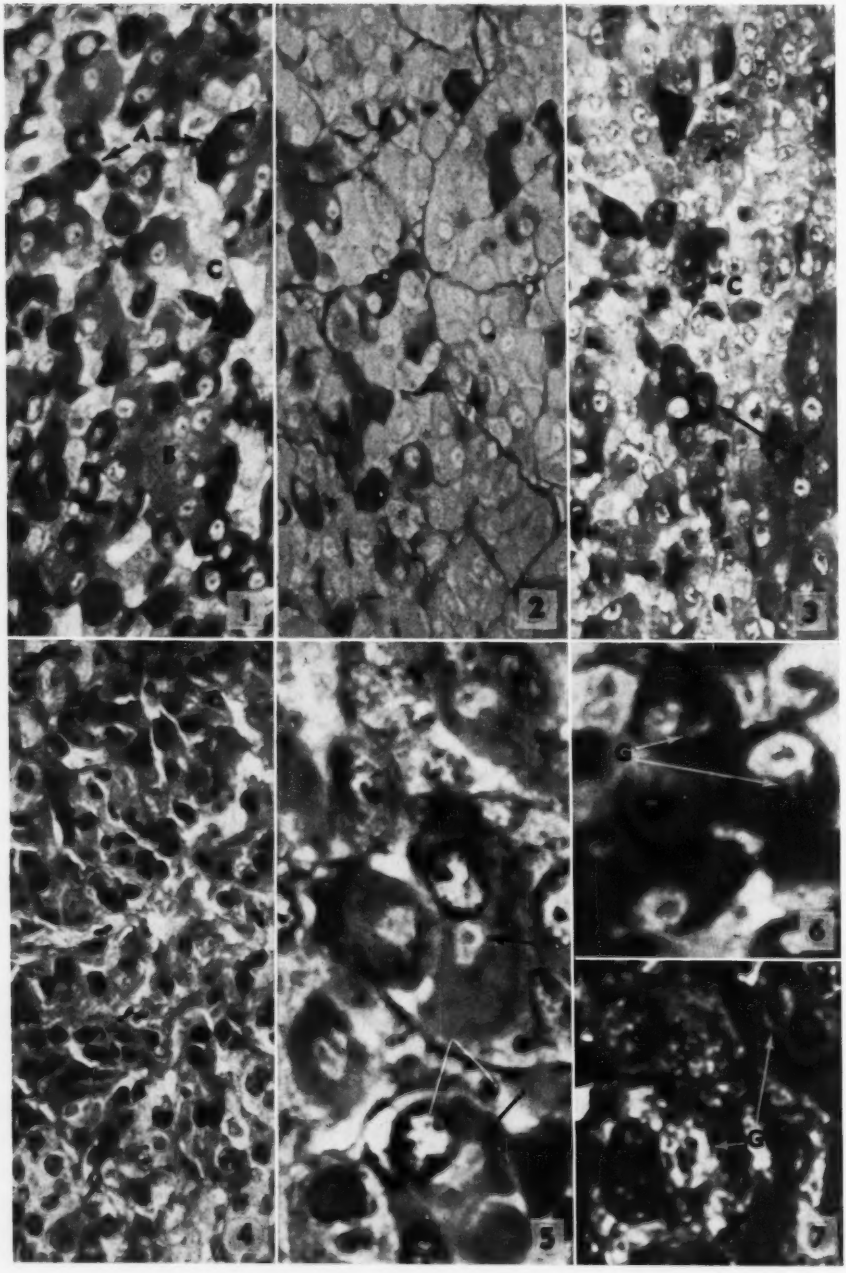
A total of 24 young adult male animals representing 5 species were used in this study; 5 cats, 4 rabbits, 4 guinea pigs, 5 rats and 6 mice. The guinea pigs, rabbits and cats were killed by an overdose of intraperitoneally injected nembutal; the mice and rats were killed by ether inhalation. The pituitary glands were removed within five minutes after death, fixed in Helly's fluid for four hours, treated with iodine and sodium thiosulfate, dehydrated in ethanol, embedded in clarite-paraffin, and serially sectioned at three microns. The sections were mounted in serial order, one section to a slide. One of several staining procedures was used after the removal of the paraffin. Controlled pH staining was done with either the acid dye, fast green, or with the basic dye, methylene blue. Dye concentration was 5×10^{-4} molar, in a solution buffered to pH values between 3.0 and 11.5 with suitable anions, in 0.1 molar concentration. The following buffers were used: dihydrogen phosphate-phosphoric acid between pH 3.0 and 3.3; acetate-acetic acid between pH 3.5 and 5.7; monohydrogen phosphate-dihydrogen phosphate between pH 6.6 and 7.6; carbonate-bicarbonate between pH 8.9 and 9.8; and tribasic phosphate-monohydrogen phosphate between pH 10.9 and 11.5. Sections were stained, without agitation, for 48 hours at room temperature, rinsed briefly in distilled water, dehydrated in three rapid changes of tertiary butyl alcohol, cleared in xylol, and mounted in Permount. Series were stained at all pH values with both acid and basic dyes, some series being stained before and some after digestion with ribonuclease. In each pH series, alternate sections were stained with the periodic acid-Schiff (PAS) procedure of McManus and Hotchkiss.

Sections to be digested with ribonuclease were placed in contact with 2 cc. of a 0.1% solution of purified crystalline ribonuclease dissolved in 0.1 molar phosphate buffer at pH 6.6 and incubated at 45° C. for 4 hours. The method of Engle and Souders (1952) was used in applying the solution to the section. Activity of the enzyme was verified by digestion of pancreatic tissue sections which had been prepared in the same way as the pituitary tissue. Control slides were incubated in buffer to which no ribonuclease had been added.

Intensity of staining with acid or basic dye was judged by eye, under the oil immersion objective, using an arbitrary intensity standard which was prepared as follows: A large sheet of thin paper was stained with either the acid or basic dye, dried and cut into strips. The strips were then mounted in a block of plastic (Bioplastic) in such a way that increasing intensities of color were obtained by increasing the number of layers of stained paper. These standards graded the intensity in equal units from one to ten plus. The standard was viewed by transmitted light and the proper intensity selected to match the intensity of staining of a given cell as seen in the microscope. Intensity of staining was then plotted against pH, the only variable, since dye concentration, buffer molarity, staining time, temperature, agitation, and section thickness, all of which may affect intensity of staining, were kept constant in these experiments. Independent observations were made by each of the authors and although there were slight differences in the staining intensities recorded for a given animal at a given pH the mean staining curve for the species was found to be the same by both authors.

OBSERVATIONS

The method of controlled pH staining with acid and basic dyes reveals, in each of the species studied, three main classes of anterior pituitary cells,



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according to their cytoplasmic and granule staining characteristics. There are two classes of granular chromophiles which stain intensely with the dyes under certain conditions. The acidophile granules stain intensely with the acid dye at low pH (Fig. 1). The basophile granules stain intensely with the basic dye at high pH (Fig. 3). A third group of cells, the chromophobes, possess a cytoplasm which stains only feebly with either acid or basic dyes (Figs. 1 and 3). Before presenting the staining characteristics of these three classes of cells in detail, it seems advisable to record certain observations on cellular organelles which are found in all the cell types. These organelles include the nucleus, the Golgi apparatus, and the ergastoplasm.

The Nucleus

In each of the species studied two types of nuclei are present, the most common being large, spherical, and vesicular with one or two nucleoli and scattered chromatin clumps; the less common being small and irregularly ovoid, with densely staining, compact chromatin (Fig. 4). The smaller, more densely staining nuclei are for the most part localized in small areas, usually at the periphery of the anterior lobe. In addition to these local concentrations, sometimes called "Kernhaufen" by the German investigators, dense nuclei are also found scattered singly throughout the rest of the gland. The cytoplasm of the densely nucleated cells is generally less abundant than is the cytoplasm of the other cells. Like the cells with large nuclei the cells with dense nuclei can be characterized as acidophiles, basophiles, or chromophobes depending upon their cytoplasmic staining characteristics.

FIG. 1. The three pituitary cell types as demonstrated by staining with the acid dye fast green at pH 3.5. Acidophiles (A) are stained intensely, basophiles (B) are moderately stained, and chromophobes (C) are unstained. Cat. ×600.

FIG. 2. Basophiles stained by the PAS reaction. Cat. ×600.

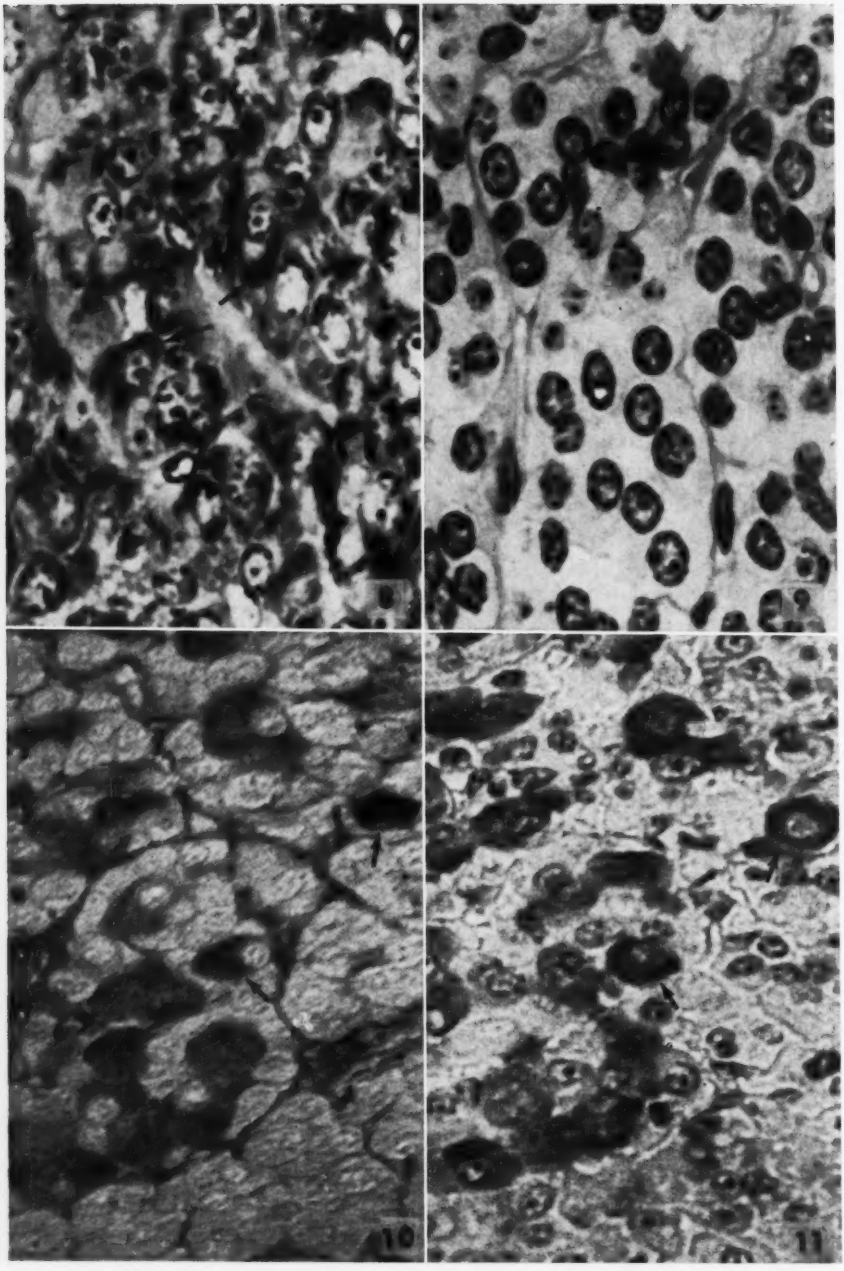
FIG. 3. The three pituitary cell types as demonstrated by staining with the basic dye methylene blue at pH 8.9. Acidophiles (A) are moderately stained, basophiles (B) are intensely stained, and chromophobes (C) are unstained. Note that both light and dark types of basophiles are shown. Cat. ×600.

FIG. 4. An area of dense nuclei. Guinea pig. Methylene blue at pH 7.4. ×600.

FIG. 5. The negative image of the Golgi apparatus (arrows) is most readily seen in basophiles that are moderately stained. Rabbit. Methylene blue at pH 6.7. ×1700.

FIG. 6. The negative image of the Golgi apparatus (G) although visible in acidophiles is partly obscured by the intense staining of granules. Rabbit. Fast green at pH 5.4. ×1700.

FIG. 7. Much more detail can be seen in the negative image of the Golgi apparatus (G) in the chromophobes when the cell is stained as intensely as possible. Contrast with that of the intensely stained basophile in the upper right corner. Rabbit. Methylene blue at pH 9.1. ×1700.



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The Golgi apparatus

In the cytoplasm of most anterior pituitary cells a juxtanuclear unstained structure is present. This is seen best in moderately stained basophiles (Fig. 5) and acidophiles (Fig. 6) and in chromophobes which are as heavily stained as possible (Fig. 7). Best visualization depends upon the presence of a cytoplasm which is homogeneous and not too heavily stained, since heavy staining may obscure the delicate features of this structure. It is seen with difficulty in the chromophobes, where the cytoplasm is not homogeneous and is usually lightly stained. When visualized, the structure is remarkably similar in the different cell types of all species. It consists of unstained lines which form a circular or irregularly ovoid halo containing transecting lines. The structure's complexity varies in degree from cell to cell, and can be seen best by using the fine adjustment of the microscope. It has been called the "macula" by early pituitary cytologists, and has been identified with the Golgi apparatus by some of them (Addison, 1917; Bailey, 1932; Kirkman, 1937) because it corresponds in position and size with the classically demonstrable Golgi apparatus of anterior pituitary cells.

The Ergastoplasm

When a section is stained with methylene blue at any pH, small cytoplasmic areas are seen in most cells which stain more intensely than the rest of the cytoplasm (Fig. 8). These areas stain with approximately the same intensity as the nuclear chromatin at all pH values. Below pH 4.0 this area alone stains in the cytoplasm. If a section is first digested with solutions, of ribonuclease, this cytoplasmic structure no longer stains with methylene blue at low pH, (Fig. 9) nor can it be recognized as an intensely stained area at higher pH. This basophilic component in the cytoplasm is rich in ribonucleic acid, and represents the ergastoplasm. The staining methods which previous investigators have used to demonstrate the ergastoplasm, such as pyronin-methyl green and toluidin blue-erythrocin, have in common, the use of a basic dye at a pH of about 3 or 3.5. Several authors have used ribonuclease to destroy the basophilia of the ergasto-

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FIG. 8. The dark areas in the cytoplasm (arrows) are typical of the basophilia of the ergastoplasm. Guinea pig. Methylene blue at pH. 5.4. ×1200.

FIG. 9. After digestion with ribonuclease the ergastoplasm no longer stains. Guinea pig. Methylene blue at pH 5.4. ×1200.

FIG. 10. Basophiles stained by the PAS reaction. Note the dark cells indicated by the arrows. Cat. ×800.

FIG. 11. The same field as in figure 10 but in the adjacent section, stained with fast green at pH 8.9. Note that the same cells are stained in each section. The cells that stain intensely with the PAS reaction also stain dark with fast green (arrows). Cat. ×800.

plasm in the pituitary (Desclin, 1940; Dempsey and Wislocki, 1945; Pearse, 1949; Abolins, 1952; Foster and Wilson 1952).

Our observations on the form and distribution of the ergastoplasm in the adenohypophysis are consistent with previous observations. In all species but the cat, the ergastoplasm is found in large amounts either centrally as perinuclear caps and wreaths, or peripherally as cytoplasmic caps (Fig. 8). This is the usual distribution in acidophiles and chromophobes. In the basophiles, however, the ergastoplasm is more commonly seen as a delicate network. Finally, in the cat ergastoplasm is rarely seen in large clumps but

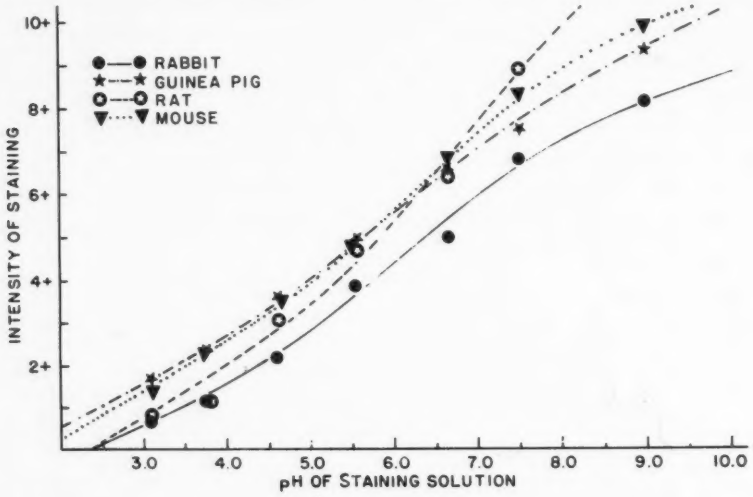

FIG. 12. Basophilia of the ergastoplasm in four species as shown by the methylene blue pH staining curves.

appears as a delicate network in all cell types. The pH staining curve of the ergastoplasm is essentially the same in the 4 species in which it is seen in clumps (Fig. 12).

Cell Types

As stated above, anterior pituitary cells can be grouped easily into one of three classes depending upon the presence and type of granules. The chromophobes appear to contain very few if any granules, and their cytoplasm stains poorly with either acid dye at low pH, or basic dye at high pH. If a section be stained with either of these procedures, the pale chromophobes stand out and can be distinguished easily from the more darkly staining granular cells (Figs. 1 and 3). Except for the ergastoplasm, the cytoplasm of the chromophobes stains so poorly with both acid and basic dyes, regardless of pH, that it is not feasible to construct pH staining curves for this cell type.

Acidophile granules are approximately half a micron in diameter, refractile when unstained, PAS negative, and stain intensely with the acid dye, fast green, at low pH. In a given individual there is slight variation in staining intensity from cell to cell, which may be related to the number of granules per cell. There is, however, greater variation in the pH staining characteristics of acidophiles from individual to individual within the same species. A plot of intensity of acidophile staining against pH for 4 individual rabbits is shown in figure 13. In each individual, staining with fast green is intense at low pH and falls off sharply between pH 5.5 and 6.5, while staining with methylene blue is only moderate even at high pH values, and completely disappears below pH 5.7. At intermediate pH

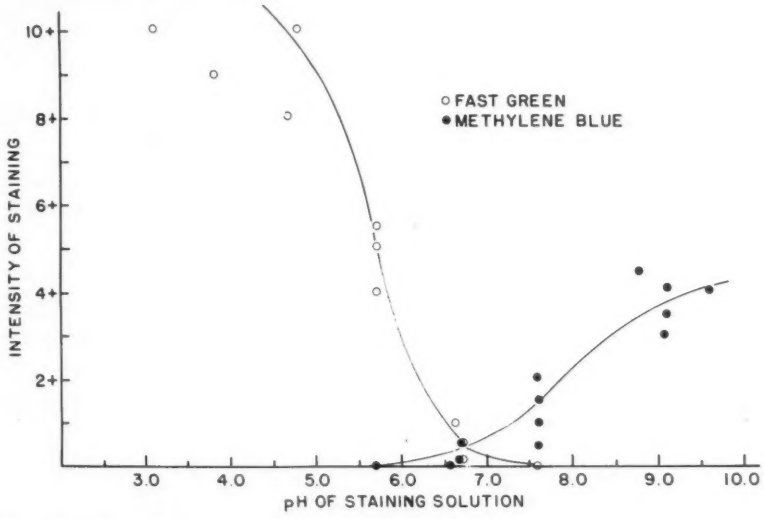

FIG. 13. pH staining curves of the acidophile cell granules in four male rabbits.

values with each of the dyes, there is moderate variation in intensity of acidophile staining from individual to individual, as is reflected by the scatter of points around the mean. To avoid confusion, only the lines representing the means are drawn for the other species in figure 14, although variation from individual to individual in each species is as great as in the rabbit. When the mean pH staining curves of acidophiles of different species are compared (Fig. 14) no significant differences can be observed.

Basophile granules are too small to be resolved by the light microscope but they can be identified by their positive PAS reaction (Figs. 2 and 10). In a single individual there is variation from cell to cell in intensity of staining with acid or basic dyes, and with the PAS reaction. In several individual cats, for example, there appear to be two main groups of basophiles, one of which stains more intensely than the other with each of

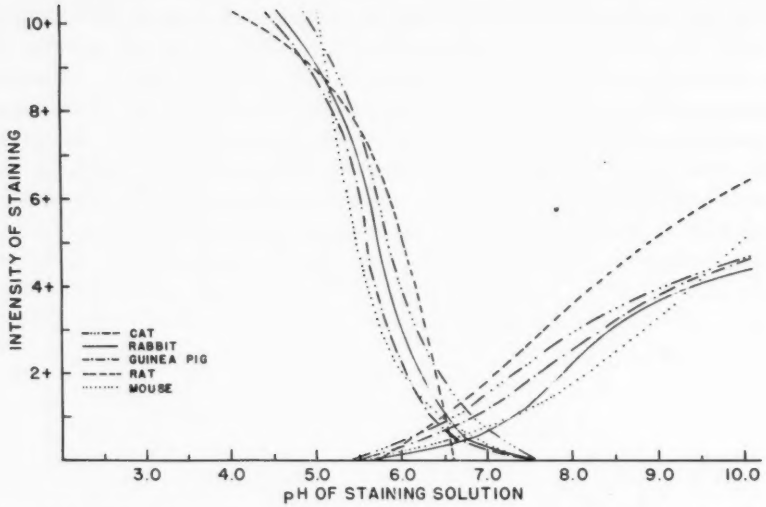

FIG. 14. pH staining curves of the acidophile cell granules in five species. The curves on the left represent the fast green staining, those on the right represent the methylene blue staining.

the staining procedures (Figs. 2, 3, 10, 11). This phenomenon is not observed in all animals, but rather, in many there is a gradual change in intensity of staining of individual cells from faint to intense. In different individuals of the same species there is moderate variation in intensity of basophile staining, illustrated in the case of the rabbit by a considerable

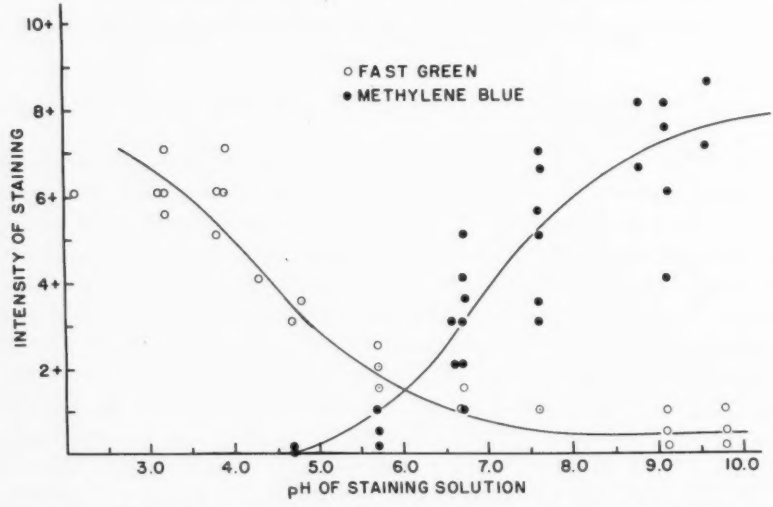

FIG. 15. pH staining curves of the basophile cell granules in four male rabbits.

scatter of individual points about the mean (Fig. 15). As in the case of the acidophiles, it is impossible on the basis of pH staining curves to distinguish between different types of basophiles in a given individual. In all species studied the basophile granules stain intensely with methylene blue at high pH and cease staining at about pH 4.5 (Fig. 16). At intermediate pH values, the basophile granules stain more intensely than the acidophile granules, but less intensely than the ergastoplasm (compare Figs. 12, 14, 16). Variation in methylene blue staining from species to species is no greater than the variation from individual to individual in the same species. The basophiles stain less intensely with fast green than the acido-

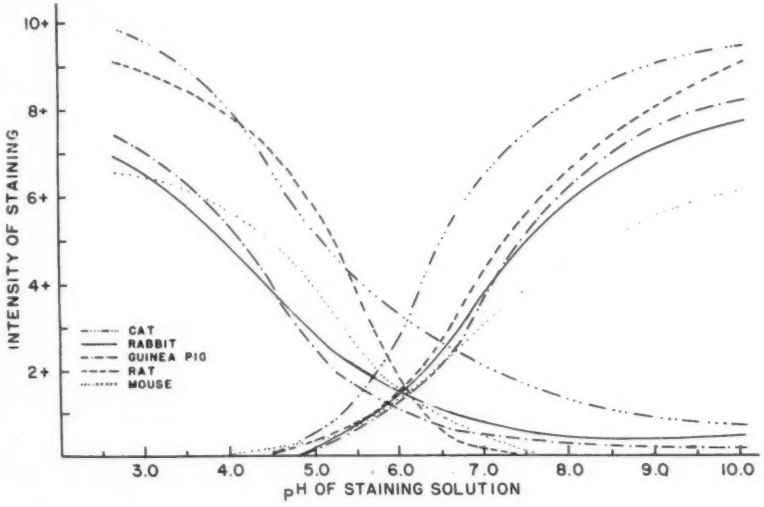

FIG. 16. pH staining curves of the basophile cell granules in five species.

philes at all pH values below 6.7 (Figs. 14 and 16). Variations in fast green staining among different species are no greater than variations from individual to individual in the same species. In the mouse and rat, basophiles do not stain with fast green above pH 7.5, even in 6 μ sections, which is double the usual thickness of sections used in this study. In the cat, rabbit and guinea pig, staining continues at very low intensity to pH values as high as 11.5. Indeed, in these species the basophiles can be specifically stained with fast green at pH 9, since at this pH nothing else but nuclei are stained (Figs. 10, 11, 17). This difference in staining is found in all individuals of these three species and is believed to represent a true species difference in the ampholyte in the basophile granules.

A graphic summary of these observations on the pH staining of acidophiles and basophiles in male rabbits is shown in figure 17. Although both cells are seen to be amphoteric in nature the acidophiles are truly more

acidophilic than the basophiles at pH values below 6.5. Similarly the basophiles are more basophilic than the acidophiles at all pH values. This can be shown in all species studied.

The staining curves of acidophile and basophile cell granules are only slightly affected, if at all, by digestion with ribonuclease. This together with the fact that these granules do not stain with methylene blue below

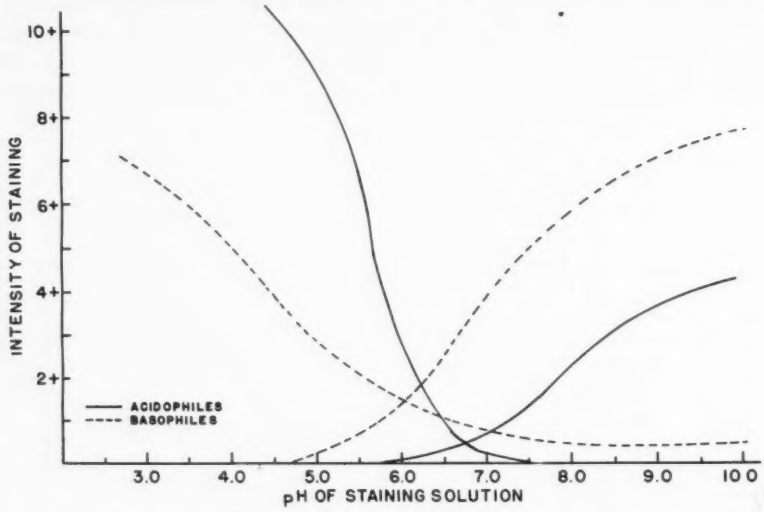

FIG. 17. pH staining curves of the acidophile and basophile cell granules in four male rabbits.

pH 4.5, provides evidence that ribonucleic acid is not present in appreciable amounts in the specific cell granules.

DISCUSSION

Much work has been done to elucidate the function of the various pituitary cell types but a confusion of terminology still persists. Goldberg and Chaikoff (1952) have described 6 cell types in the adenohypophysis of the dog designating them alpha through zeta following the terminology used by Romeis (1940). Purves and Griesbach (1951) divided the PAS positive cells into thyrotrophs and gonadotrophs. Halmi (1952) has called these same cells beta and delta respectively.

The trend toward abandonment of the terms acidophiles and basophiles was started because it seemed unreasonable to name a cell that is usually demonstrated by staining with the acid dye, anilin blue, a basophile. The reason for the selective staining by anilin blue after mordanting is still enigmatic. One of us (Peterson, unpublished data), in a preliminary investigation of the pH staining characteristics of anilin blue, confirmed that it does behave like the acid dye fast green in that it stains the pituitary

more intensely at low pH values than at higher pH, but unlike fast green, it stains the basophiles more intensely than the acidophiles at each pH. After mordanting with phosphotungstic acid the basophiles and connective tissue are the only elements which stain. The explanation for this peculiar phenomenon is now being investigated. One factor in the behavior of this dye may be that basic groups, as well as acid groups are present in the complex molecule.

Still another factor causing dissatisfaction with the nomenclature has been that the basophilic ergastoplasm has been confused with the basophile cell granules. Benda (1900) was the first to describe basophilic Nissl-like inclusions in certain granular and agranular cells which were probably the same structures identified as ergastoplasm by da Costa (1923). Kirkman (1937) described "Nebenkern-like" objects and filamentous ergastoplasmic elements in the acidophiles of guinea pigs. We have found ergastoplasm in all cell types of all species studied. The morphology of the ergastoplasm is similar in all these species except the cat, in which the clumps usually found in acidophiles and chromophobes are replaced by a delicate network. This organelle is more basophilic than any other cytoplasmic substance in the pituitary, including the basophile granules. This basophilia was shown by Desclin (1940) to be due to ribonucleic acid by utilizing the then newly developed techniques of Brachet to show that the ergastoplasm did not stain after ribonuclease digestion. We have found that the basophilia of the basophile cell granules, on the other hand, is not affected by ribonuclease digestion. This is consistent with the observations of Foster and Wilson (1952) that the PAS and Gram reactions of the basophile cell granules are not affected by prior digestion with ribonuclease. The changes in the basophilia of the basophile cell granules after ribonuclease digestion which have been reported by Dempsey and Wislocki (1945) and by Pearse (1949) possibly resulted from a confusion of the basophilic ergastoplasm, found in all cell types with the basophile cell granules, found in only one class of cells.

A consideration of our findings in the light of what has been stated above leads us to believe that until there is final definition of pituitary cells according to function, with clear and easily applied tests or stains for each type, it would be well to retain a simple nomenclature consistent with the known facts. It is our opinion that the acidophile, basophile, chromophobe nomenclature introduced by Thom (1901) is informative and should be retained.

SUMMARY AND CONCLUSIONS

The method of controlled pH staining with acid and basic dyes was applied to the adenohypophyses of male mice, rats, guinea pigs, rabbits and cats. It has been found that the three classical cell types, acidophiles, basophiles and chromophobes have distinctly different affinities for acid

and basic dyes. The acidophiles are stained intensely by acid dye at low pH, and only moderately stained by basic dye at high pH. The basophiles are stained intensely by basic dye at high pH and moderately stained by acid dye at low pH. Thus it is possible on the basis of pH staining to distinguish between acidophiles and basophiles even though both types of cell granules are amphoteric staining with either acid or basic dye. The agranular chromophobes are stained very feebly by either acid or basic dye only at extremes of pH.

Ribonuclease digestion in conjunction with pH staining shows that the basophilia of the basophiles is distinct from that of the ergastoplasm. The ergastoplasm is stained by the basic dye to a much lower pH than the basophile granules. The ergastoplasm is present in all cell types and its basophilia is eliminated by ribonculease digestion, whereas the basophilic granules are found only in certain cells and their basophilia is unaffected by the digestion. If follows that the cell granules do not contain appreciable amounts of ribonucleic acid.

The species comparison shows that the staining characteristics of the acidophiles and chromophobes are remarkably constant. Species differences in the staining characteristics of the basophiles are noted. Only in the guinea pig, rabbit and cat are these cells selectively, though weakly stained by acid dye at high pH.

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LIVER CATALASE ACTIVITY IN PARABIOTIC RATS, ONE OF WHICH IS THYROIDECTOMIZED[1]

ISOLDE T. ZECKWER AND MARY BERWICK

Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

THE fact that liver catalase activity is reduced in tumor-bearing animals has led to many investigations, but the meaning of this change has not yet been elucidated (Greenstein 1954). Because it seemed important to know more about what factors operate in regulating catalase activity in non-tumor-bearing animals, we have investigated further the role of endocrine factors.

Liver catalase activity is lowered by adrenalectomy and by castration of male mice (Adams 1952). On the other hand, an increase in catalase activity of the liver has been reported after hypophysectomy (Gaebler and Mathies 1951). In our previous experiments we found an increase in liver catalase activity after thyroidectomy in rats and after thiouracil administration in rats and mice (Zeckwer, Berwick, Lucké 1954).

The present experiments were carried out to see if the method of parabiosis would give a clue as to the mechanism by which hypothyroidism results in an increase in catalase activity.

METHODS

White rats were thyroidectomized and sufficient time was allowed for loss of circulating thyroid hormone and for the development of histological changes in the pituitary. Time intervals were based on our previous experience (Zeckwer, Davis, Keller, Livingood 1935). Then parabiotic union was made surgically between a thyroidectomized rat and a litter-mate normal rat of the same sex. A strain of white rats was used that had been inbred in this laboratory. The need for using inbred rats for parabiosis has been emphasized by Van Dyke and Huff (1949). This avoided the incompatibilities that we had encountered when rats are used that are not closely related (Zeckwer 1952).

In each experiment a group of 4 rats was used: a parabiotic pair, one partner of which had been thyroidectomized; a single rat thyroidectomized at the same time as the parabiont; and a single intact rat. The single control rats were often litter-mates of the parabiotic rats. A total of 60 rats was employed, consisting of 10 groups of males and 5 groups of females. Fourteen to 28 days were allowed to elapse after the parabiotic operation in order to insure a good circulation, and to allow time for the rats to become adapted to the parabiotic union. Our previous experiments (Lucké, Berwick, Zeckwer

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1952) indicated that the parabiotic operation per se caused no significant change in catalase activity, so that this control was not needed in the present experiments. The rats were killed at ages varying from 143 to 341 days for the males, 407 to 509 days for the females, and 50 to 113 days after thyroidectomy.

Catalase activity was determined by the method previously used (Zeckwer, Berwick, Lucké 1954), and is expressed as "K"/0.02 mg. N.

Histological sections of the pituitaries showed that the degree of hypothyroidism was sufficient to cause thyroidectomy changes in the basophilic thyrotrops.

RESULTS

The data are given in Tables 1 and 2. It will be seen that the male thyroidectomized rats showed significant increase in liver catalase activity,

TABLE 1. LIVER CATALASE ACTIVITY IN MALE RATS

Group	"K"/0.02 Mg. N.			
	Single thyroidectomized rats	Parabiotic rats		Single normal rats
		Thyroidectomized partners	Normal partners	
1	.779	.732	.676	.600
2	.727	.844	.718	.570
3	.777	.710	.678	.655
4	.843	.880	.701	.599
5	.717	.762	.730	.615
6	.834	.752	.705	.495
7	.818	.723	.644	.442
8	.740	.789	.740	.591
9	.759	.708	.618	.610
10	.901	.870	.830	.604
Mean $\bar{x}$	.790	.777	.704	.579
Standard Error, s	.058	.065	.041	.045

"T" tests according to Snedecor (1946).
Between single normal rats and thyroidectomized single rats: $t = 7.96$, $p < 0.01$, 18 d.f.
Between single thyroidectomized rats and parabiotic thyroidectomized rats: $t = 0.48$, $p > 0.05$, 18 d.f.
Between single normal rats and parabiotic normal rats: $t = 4.61$, $p < 0.01$, 18 d.f.
Between parabiotic normal rats and thyroidectomized partners: $t = 2.64$, $p < 0.02$, > 0.01, 18 d.f.

whether they were single or in parabiosis. That is, the normal rats did not affect the catalase activity of the thyroidectomized partners. The normal rats united with the thyroidectomized rats showed a rise above normal single rats. Thus the thyroidectomized parabionts had a pronounced effect upon the normal partners, but the increased catalase activity of the normal partner was never as great as in the thyroidectomized partner.

The results in the female rats gave such widely scattered values that no significant conclusions could be drawn. However, the trend was in the same direction as for male rats. Probably estrous cycles introduced complicating factors. The presence or absence of gonadal hormones have been found to alter catalase activity (Adams 1951, 1952).

TABLE 2. LIVER CATALASE ACTIVITY IN FEMALE RATS

Group	"K"/0.02 Mg. N.			
	Single thyroidectomized rats	Parabiotic rats		Single normal rats
		Thyroidectomized partners	Normal partners	
1	.654	.812	.651	.498
2	.742	.691	.683	.553
3	.679	.813	.788	.657
4	.690	.502	.469	.374
5	.620	.555	.274	.264
Mean $\bar{x}$	.677	.674	.573	.469
Standard error, s	.045	.139	.202	.119

DISCUSSION

The discussion will be confined to the observations made on the male rats.

It has been demonstrated repeatedly that substances pass across the parabiotic union when in high concentration provided that these substances are not quickly utilized, excreted, or destroyed in the animal producing them. In our previous experiments (Lucké, Berwick, Zeckwer 1952), after rats were united in parabiosis, an implanted tumor in one rat resulted not only in a fall in catalase activity in that rat, but also a fall in the normal partner. This was interpreted as due to the passage of a high level of catalase inhibiting substance or substances, with low clearance rate, across the parabiotic union.

The results of the present experiments suggest that in the thyroidectomized rat there is an excess of some substance with low clearance rate, which is present in the circulating blood. In much of the work on catalase activity in tumor-bearing animals, the emphasis has been placed on the production by the tumor of an inhibitor. Recently Day, Gabrielson and Lipkins (1954) have shown that in the spleen of normal mice there is a catalase inhibitor. In the normal animal, the catalase activity at any given time may be conceived of as a balance between catalase produced and an inhibitor of catalase. After thyroidectomy it is conceivable that both factors might be reduced rather than that inhibitor alone is reduced. In either case, the ratio of catalase production to inhibitor is altered in such a way that catalase activity predominates over inhibition. This relative increase in available catalase may then pass to the normal partner.

In a parabiotic union between a thyroidectomized rat and a normal rat, the uninhibited secretion of pituitary thyrotropic hormone (TSH) by the thyroidectomized rat passes to the normal partner (Koneff, Van Dyke and Evans 1952) and causes increased secretion of its thyroid. It would be anticipated that any increased secretion of thyroid hormone by the normal partner would be quickly utilized by that animal's tissues, but

some thyroid hormone passes to the thyroidectomized partner, as indicated by the experiments of Griesbach and Kennedy (1949) and Koneff, Van Dyke and Evans (1952), who found less extensive histological changes in the pituitary of thyroidectomized rats united with normal rats as compared with single thyroidectomized rats. The same differences in histological appearance were noted in our experiments, and yet there was no significant difference in liver catalase activity in the thyroidectomized rats united with normal rats as compared with single thyroidectomized rats.

If the thyroid in some way, directly or indirectly, promotes inhibition of catalase activity, then removal of the thyroid should remove the inhibition and thereby release catalase. The stimulated thyroid of the normal parabiont may promote some inhibition of the catalase transported from the thyroidectomized partner, and would account for the fact that the normal partner does not have as great an increase in catalase activity as does the thyroidectomized partner.

As suggested in our previous experiments (Zeckwer, Berwick, Lucké 1954) the effect of thyroidectomy may be due to hypofunction of the anterior pituitary secondary to hypothyroidism, rather than to the absence of the thyroid per se. The argument for this hypothesis is that hypophysectomy causes greater increase in liver catalase activity than does thyroidectomy. If it is the pituitary that is responsible for catalase inhibition, then the effect of surgical removal of the pituitary would be greater than the effect of any hypofunction of the pituitary secondary to hypothyroidism. It would be of interest to determine the catalase activity in parabiotic rats, one of which is hypophysectomized.

Recent experiments (Melchior and Sliwinski 1954) indicate that another enzyme is inhibited by the pituitary. Serum antitryptic activity was lowered by hypophysectomy, whereas pituitary extract administration reversed the effect.

SUMMARY

Liver catalase activity was increased beyond the normal range in male thyroidectomized rats united in parabiosis with normal partners. This increase was of the same degree as in thyroidectomized single rats, indicating that the normal partner had not influenced the thyroidectomized parabiont. The normal parabiont showed an increase in catalase activity above the values for single normal rats, indicating that some substance had passed from the thyroidectomized rat across the parabiotic union to the normal partner. It is suggested that the thyroidectomized rat has less catalase inhibitor so that a relative preponderance of catalase results. This relative increase is evidently transferred to the normal partner.

These results suggest that the thyroid in some way promotes inhibition

of catalase activity, and in the absence of the thyroid, this inhibition is depressed. The effect of thyroidectomy on catalase activity may be the result of hypofunction of the pituitary secondary to hypothyroidism.

Since the normal partner is receiving increased secretion of thyrotropic hormone from the thyroidectomized partner, as shown in the experiments of others, the increased thyroid activity of the intact partner may partially inhibit the transferred catalase and thus account for the fact that the normal partner does not show as great an increase in catalase activity as does the thyroidectomized partner.

Results in female rats were too scattered to permit drawing any conclusions, probably because estrous cycles introduced complicating factors, but the trend was in the same direction as in the males.

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PITUITARY-ADRENAL FUNCTION DURING PSEUDOPREGNANCY AND EARLY PREGNANCY IN RATS[1]

NEENA B. SCHWARTZ, LOIS E. SHIRKY AND IRVING E. STECK

Departments of Physiology and Medicine, University of Illinois College of Medicine, Chicago

NUMEROUS studies have demonstrated an increase in adrenal-like function during pregnancy. Pregnancy and pseudopregnancy increase the survival time of adrenalectomized mammals (Firor and Grollman, 1953; Rogoff and Stewart, 1927; Swingle, *et al.* 1937). During late pregnancy adrenal hypertrophy occurs in rats (Mutow, 1937), guinea pigs (Hewitt and Van Liere, 1941) and rabbits (Randall and Graubard, 1940). In human beings during pregnancy, an increased urinary corticosteroid excretion (Venning, 1946) and an increased blood level of 17-hydroxycorticosterone (Gemzell, 1953) occur, even in Addisonian patients (Knowlton, *et al.*, 1949), and there is often an amelioration of rheumatoid arthritis (Hench, 1938).

The extent to which the fetal adrenal-pituitary system and the maternal placenta are responsible for the above findings is not completely known. The fetal pituitary probably secretes ACTH since decapitation causes fetal adrenal atrophy (Wells, 1948) and unilateral adrenalectomy induces hypertrophy in the remaining adrenal (Kitchell, 1950). Adrenalectomy of the pregnant rat induces fetal adrenal hypertrophy (Ingle and Fisher, 1938) and cortisone injections into the mother cause fetal adrenal atrophy (Davis and Plotz, 1954). Since adrenalectomy in the hypophysectomized mother does not cause fetal adrenal hypertrophy (Knobil and Briggs, 1954) maternal ACTH must be able to cross the placental barrier, although Jones, *et al.* (1953) claim that this occurs only when the maternal ACTH levels are very high. Adrenocorticotrophic (Assali and Hamermesz, 1954) and corticoid (Johnson and Haines, 1952) activity have been isolated from the human placenta. In the mouse (Gardner and Allen, 1942) and rat (Greer, 1949) late pregnancy does not seem to prevent the adrenal atrophy or histochemical changes which occur following hypophysectomy, suggesting that the placenta (and fetal pituitary) cannot completely replace the adrenocorticotrophic function of the maternal pituitary.

The present investigation was undertaken in an attempt to isolate

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maternal pituitary-adrenal function from possible fetal and placental participation. Adrenal weights and ascorbic acid concentrations were measured on the 12th day of pregnancy and pseudopregnancy, in intact and unilaterally adrenalectomized rats. The rat fetal adrenal does not develop until the 13th day (Pankratz, 1931) and probably does not secrete hormones much before the 19th day (Josimovich, *et al.*, 1954), thus eliminating the fetal adrenal from consideration. Pseudopregnant rats were used to eliminate placental hormones as a factor. Since the maternal pituitary is indispensable in the rat for maintenance of the uterus during the first 11 days of pregnancy (Pencharz and Long, 1933), this study, in addition to providing a background for an investigation of adrenal-pituitary relationships in later pregnancy, also supplies information on the ability of the pituitary to secrete ACTH concurrently with its secretion of gonadotrophic hormones.

MATERIALS AND METHODS

Ninety-day-old virgin female rats were obtained from Sprague-Dawley, Inc. in two separate lots, for the two experiments described below. The rats were kept in a constant temperature room, artificially lighted from 5:00 A.M. to 7:00 P.M. Daily vaginal smears were taken in the morning and all rats were running 4 to 6-day cycles before the experiment began. *Pseudopregnancy Experiment.* Eighteen rats were mated with vasectomized males, mating being detected by the presence of a copulation plug. Of these, ten rats (Group IV) were anesthetized with sodium pentobarbital (5 mg./100 g.) on the 2nd day of pseudopregnancy, and the left adrenal removed. Ten days later, the rats were decapitated and the right adrenal removed. The remaining eight pseudopregnant rats (Group II) were decapitated on the 12th day of pseudopregnancy, and both adrenals removed. Seven non-pseudopregnant rats (Group III) were decapitated on the same days as rats of Group II, both adrenals being removed. Seven non-pseudopregnant rats (Group I) were unilaterally adrenalectomized on the same days as rats of Group IV and ten days later were decapitated, and the right adrenal removed. The ascorbic acid content and wet weight were measured on all adrenals removed. Body weights were measured for all rats at the time of death, and for the unilaterally adrenalectomized rats also on the day of surgery.

Pregnancy Experiment. Essentially, the above experiment was repeated, the mating taking place with normal males in the experimental rats. Two changes in data accumulation were made: (1) body weights of all the rats were measured on the first day of pregnancy (non-pregnant rats randomly matched to pregnant rats on this day) and on the day of death; (2) adrenal ascorbic acid content was measured only on the right adrenals.

Ascorbic Acid Measurements. The method of Roe and Keuther (1943) as adapted by Sayers, *et al.*, (1948) was used, with two modifications: (1) a wave length of 510λ, instead of 540λ, was found to give minimal transmittance and a more sensitive standardization curve and accordingly was used; (2) the large size and ascorbic acid content of adult female adrenals necessitated using 20 cc. of 4% trichloroacetic acid for each gland, a 4 cc. aliquot being used for chemical measurement.

RESULTS

Adrenal Weights. On the second day of pseudopregnancy (Table 1) left adrenal weight was not different from control values (III vs. IV) whereas,

TABLE 1. ADRENAL WEIGHT AND ASCORBIC ACID CONCENTRATIONS IN PSEUDOPREGNANCY

Group	Day	No.	Body weight‡	Adrenal weight			Adrenal ascorbic acid	
				Left	Right	Left	Left	Right
			g.	*mg.*	*mg.*	*mg./100 g.*	*μg./mg.*	*μg./mg.*
I. Control BA*	0	7	288.0 ±23.5	41.4 ±5.6	38.9 ±5.5	14.4 ±1.5	4.61 ± .54	4.37 ± .38
II. Pseudopreg. BA*	12	8	299.7 ±19.4	36.2 ±4.5	33.7 ±3.9	12.2 ±1.7	4.33 ± .52	4.38 ± .37
III. Control UA*	0	7	278.8 ±21.6	39.2 ±3.9	—	14.2 ±2.0	3.65 ± .73	—
	10	7	284.3 ±27.5	—	45.2 ±3.5	—	—	4.24 ± .37
IV. Pseudopreg. UA*	2	10	287.0 ±16.6	40.6 ±3.6	—	14.2 ±1.4	3.70 ± .44	—
	12	10	299.8 ±17.6	—	39.4 ±3.3	—	—	4.35 ± .49

All values mean ± standard deviation.
* BA = Bilaterally adrenalectomized; UA = unilaterally adrenalectomized.
‡ Body weight at time of adrenal removal.

on the 12th day significant atrophy of the adrenal glands had taken place (I vs. II). The absolute weight of the right adrenals in the pseudopregnant rats, unilaterally adrenalectomized on the second day, was significantly lower than that of control rats unilaterally adrenalectomized ten days previously (III vs. IV).

TABLE 2. ADRENAL WEIGHT AND ASCORBIC ACID CONCENTRATIONS IN EARLY PREGNANCY

Group	Day	No.	Body weight‡		Adrenal weight			Adrenal ascorbic acid
			Begin	End	Left	Right	Left	Right
			g.	*g.*	*mg.*	*mg.*	*mg./100 g.*	*μg./mg.*
I. Control BA*	0	8	243.8 ±15.4	248.0 ±14.1	36.3 ±3.3	33.6 ±2.6	14.1 ±2.0	4.65 ± .24
II. Pregnant BA*	12	8	239.0 ±11.8	270.6 ±15.0	34.7 ±4.3	32.4 ±4.4	12.8 ±1.1	4.58 ± .28
III. Control UA*	0	8	248.6 ±15.9	—	36.7 ±4.5	—	14.8 ±1.3	—
	10	8	—	246.3 ±19.3	—	39.9 ±4.6	—	4.53 ± .56
IV. Pregnant UA*	2	8	239.9 ±15.3	—	36.3 ±3.8	—	15.1 ±1.3	—
	12	8	—	259.6 ±17.6	—	38.8 ±4.7	—	4.55 ± .35

All values mean ± standard deviation.
* BA = bilaterally adrenalectomized; UA = unilaterally adrenalectomized.
‡ "End" is body weight when either both adrenals were removed (BA) or the right adrenal removed (UA). "Begin" body weight was the body weight 12 days earlier.

Adrenal weights were lower in the controls used in the pregnancy experiment (Table 2) than in those used in the previous experiment. This is apparently a reflection of the lower body weights, since the adrenal weight is the same fraction of body weight in both sets of controls. No statistically significant change in the *absolute* adrenal weight on either the 2nd or 12th day of pregnancy was noted (Table 2); however, adrenal weight tended to be a smaller fraction of body weight on the 12th day of pregnancy. Moreover, the weight of the right adrenal gland in unilaterally adrenalectomized controls and pregnant animals was the same.

"Compensatory" Hypertrophy. Calculation of the "compensatory" hypertrophy of the right adrenal glands was complicated by the atrophy of the glands in the previously untreated pseudopregnant rats (Group II), and by the consistent difference in weight between left and right adrenals

TABLE 3. CALCULATION OF RIGHT ADRENAL HYPERTROPHY

Experiment	Group	Right adrenal wt., mg.		Hypertrophy	
		Expected (BA)	Actual (UA)	Mg.	% of expected
Pseudopregnancy	Control	38.9	45.2	6.3	16.2
	Pseudopregnant	33.7	39.4	5.7	16.9
Pregnancy	Control	33.6	39.9	6.3	18.7
	Pregnant	32.4	38.8	6.4	19.8

seen in all groups. It seemed reasonable to assume that had the left adrenals not been removed, the right adrenals would have weighed the same as the right adrenals in the respective bilaterally adrenalectomized group. Accordingly the calculation of hypertrophy was made (Table 3), using only group averages. On the basis of this assumption, the hypertrophy of the right adrenal was the same in all four groups of rats.

Ascorbic Acid Concentrations. No differences in ascorbic acid concentrations among the treatment groups were demonstrable. The slight depletion of ascorbic acid in the left adrenals of groups III and IV (Table 1) was undoubtedly due to the effects of anesthesia and laparatomy.

DISCUSSION

Since the degree of "compensatory" hypertrophy was unaltered from control values during early pregnancy and pseudopregnancy, it is probable that the amount of ACTH released by the pituitary in response to unilateral adrenalectomy is normal, indicating that this secretion is unaltered by concomitant secretion of gonadotrophins. In pregnant animals, unstressed by unilateral adrenalectomy, absolute adrenal weight and ascorbic acid concentration are normal on the 12th day, suggesting normal levels of ACTH in the blood. This confirms previous work on adrenal weight during early pregnancy (Mutow, 1937).

Although Andersen and Kennedy (1933) failed to show significant adren-

al weight changes (in four rats) on the 7th day of pseudopregnancy, the present work indicates that adrenal atrophy has occurred by the 12th day. The most probable explanation of this change is a decrease in output of ACTH from the pituitary. At least two explanations may be offered to account for the apparent difference in "basal" level of ACTH secretion in pregnant and pseudopregnant rats:

(1) In both pregnancy and pseudopregnancy there may be low ACTH output by the pituitary because of high progesterone levels (Selye, 1940), but placental ACTH secretion may substitute for this in the pregnant animal.

(2) There may be some hitherto unsuspected difference between the endocrinology of early pregnancy and pseudopregnancy not related to possible placental ACTH production. Perhaps progesterone levels are higher in pseudopregnancy, because of lack of "utilization" by decidual tissue.

At present, the foregoing discussion is highly speculative.

One remaining point for consideration is the separation, in the pseudopregnant rats, between "basal" ACTH production (which was low) and ACTH production occurring in response to unilateral adrenalectomy (which was normal). This finding is similar to the observation (Winter and Emery, 1936) that in the sprayed female rat adrenal atrophy occurs but adrenal hypertrophy in response to unilateral adrenalectomy is normal. These data support the suggestion (Ganong and Hume, 1954) that "basal" ACTH production may be controlled by a different mechanism than the production in response to a stress or unilateral adrenalectomy.

SUMMARY

Adrenal weight is normal on the 12th day of pregnancy but reduced significantly on the 12th day of pseudopregnancy in rats. Adrenal ascorbic acid concentrations are normal in both pregnancy and pseudopregnancy on the 12th day. Removal of the left adrenal gland on the 2nd day of either pregnancy or pseudopregnancy induces hypertrophy of the right adrenal (measured on the 12th day) of the same magnitude as in non-mated control rats.

The results indicate a lower ACTH output in pseudopregnancy than in pregnancy. They also suggest that the secretion of gonadotrophins during pregnancy or pseudopregnancy does not alter the ability of the pituitary to secrete ACTH in response to unilateral adrenalectomy.

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RELATIONSHIP OF VITAMIN B_{12} TO STILBESTROL STIMULATION OF THE CHICK OVIDUCT[1]

IRENE T. KLINE

Departments of Obstetrics and Gynecology, and Biochemistry, University Hospitals and Western Reserve University School of Medicine, Cleveland, Ohio

RESPONSE of the chick oviduct to stilbestrol is severely limited by a deficiency of pteroylglutamic acid (PGA), while in other vitamin deficiency states investigated, little or no impairment of oviduct response to stilbestrol is observed (Hertz and Sebrell, 1944; Hertz, 1945; Franklin *et al.*, 1948; Kline and Dorfman, 1948, 1951a, b).There is, furthermore, a quantitative relationship between the availability of PGA and the response of the chick oviduct to stilbestrol, whether the availability be regulated by dietary intake or by administration of specific antagonists such as aminopterin. Protection against aminopterin interference with stilbestrol stimulation of the chick oviduct is afforded by PGA given one hour prior to aminopterin (Hertz, 1948; Hertz and Tullner, 1949; Kline and Dorfman, 1951b). More efficient than PGA in preventing aminopterin inhibition of stilbestrol action is the citrovorum factor (CF), a metabolic conversion product of PGA (Kline and Dorfman, 1951b).

An intimate functional relationship, which has not yet been elucidated, exists between PGA and vitamin B_{12} (Welch and Nichol, 1952; Jukes and Williams, 1954). Both vitamins have been implicated in the intermediary metabolism of one carbon units but their individual roles, particularly that of vitamin B_{12}, are still obscure. The work of Greenberg (1954a, b), of Kisliuk and Sakami (1954), Sakami (1955), and of others (as reviewed by Welch and Nichol, 1952; Jukes and Williams, 1954), indicates that one function of PGA, in a metabolically active form, is the transport and activation of one carbon units in different states of oxidation. From the work of Arnstein and Neuberger (1953), of Kratzer (1953), of Stekol's group (1953a, b), and of Young, Norris and Hauser (1954), since these workers present evidence that vitamin B_{12} is essential to the synthesis of methyl groups *de novo*, there is a possibility that vitamin B_{12} is involved in the reduction of certain one carbon units to methyl groups. (If such reduction be stepwise, this hypothesis would imply a role of vitamin B_{12}, as well as

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of PGA, in the synthesis of purines via the formation of Greenberg's transformylation coenzyme.) Transmethylation reactions, which involve preformed methyl groups, do not require vitamin B_{12}, however, either *in vivo*, according to the investigators just mentioned, or *in vitro* according to Mistry *et al.* (1954). Jukes and Stokstad (1952), on the other hand, have presented data which they interpret as evidence for the dependence of transmethylation on vitamin B_{12}. It has also been suggested that vitamin B_{12} acts catalytically to promote the conversion of PGA to CF (Welch and Nichol, 1952; Jukes and Williams, 1954). These experimental results, however, are very difficult to interpret and seem to be inconclusive and conflicting.

Previous reports on the relation of vitamin B_{12} to estrogen action have been concerned with the effect of supplementary vitamin B_{12} in animals maintained on diets generally considered to be adequate. Feeding stilbestrol severely inhibited appetite and growth in male rats (Meites and Shay, 1951; Meites, 1952). The inhibition was partially overcome by additional vitamin B_{12} but not by additional PGA. Vitamin B_{12} in the doses used, however, did not prevent the reduction in weight of testes seen in the stilbestrol-fed rats nor alter the ability of the stilbestrol to increase the weight of the seminal vesicles. In prepuberal, ovariectomized rats, uterine response to estradiol benzoate was increased by supplementary PGA or CF, but not by supplementary vitamin B_{12} (Penhos, 1953). Addition of vitamin B_{12} to a commercial mash diet resulted in significantly increased body weight and oviduct response to stilbestrol in a heavy breed of chick (Sex-Linked Cross), but not in a light breed (White Leghorn) (Kline and Dorfman, 1951a). Vitamin B_{12} added to a synthetic casein diet did not result in increase in body or oviduct weights in White Leghorn chicks given stilbestrol, but in Sex-Linked Cross chicks, had the effect of increasing body weight and decreasing oviduct response to stilbestrol (Kline and Dorfman, 1951a).

Because of the requirement of PGA for optimal response of the oviduct to estrogen in pullets and because of the functional relationship between PGA and vitamin B_{12}, it seemed possible that vitamin B_{12} might also be essential for optimal stimulation of the chick oviduct. Responsiveness to stilbestrol of pullets deficient in vitamin B_{12} has, therefore, been investigated. It also seemed possible to test the hypothesis that vitamin B_{12} catalyzes the conversion of PGA to CF, or other metabolically active products, by quantitating in vitamin B_{12}-deficient, aminopterin-treated pullets, the stilbestrol-stimulated oviduct growth permitted by minimal amounts of PGA in the presence and in absence of supplementary vitamin B_{12}.

ANIMALS AND DIET

The chicks used were made available through the courtesy of Dr. L. C. Norris from the flock of vitamin B_{12}-depleted White Leghorn hens at the Agricultural Experiment

Station of Cornell University. On arrival in the laboratory the chicks were sexed and the pullets used in these experiments. They were housed in thermostatically controlled, electrically heated brooders with raised wire mesh floors. The floors and water troughs were scrubbed daily and were rinsed first in a cresol solution and then thoroughly in hot running water. Each shipment of chicks was received in the laboratory the day after hatching, hereafter designated as day one.

Since the chicks were made available when hatchability was very low, the additional depletion period required was brief but the small number in any one hatch limited the control groups which could be included in any one experiment.

The diet used in all experiments, except Experiment 1M in which the chicks were starved, consisted of a basal purified ration (available *ad libitum*) and an oral, aqueous vitamin supplement which together provided adequate amounts of all nutrients known to be required by the chick except vitamin B_{12}, PGA, methionine and choline. The basal ration was constituted as follows: alpha protein,[2] 25 gm. (88% protein, 1.6% methionine, and 1% Na_2SO_3 as SO_2); gelatin, 10.0 gm.; L-cystine, 0.3 gm.; choline chloride, 0.1 gm.; corn starch, 52.4 gm.; Cellu flour, 3.0 gm.; Crisco, 4.0 gm.; salts (Briggs *et al.*, 1943), 5.0 gm.; manganous sulfate, 0.1 gm.; alpha tocopherol, 0.025 gm.; vitamin A, 1800 U.S.P. units; and vitamin D_3, 180 units. The vitamin supplement was made up to provide in the 0.1 ml. dose given orally, daily, the following: Ca-pantothenate, 74 μg.: riboflavin, 60 μg.; nicotinic acid, 125 μg.; i-inositol, 2.5 mg.; thiamine chloride, 50 μg.; pyridoxine hydrochloride, 50 μg.; 2-methyl-1, 4-naphthoquinone, 0.5 μg.; and biotin, 1.5 μg. Additional thiamine chloride, 260 μg. per 0.1 ml. of water, was injected daily to compensate for the destruction of thiamine in the gut by the sodium bisulfite. Details of dosages and treatment schedules for vitamin B_{12}, PGA, stilbestrol and aminopterin are given in the footnotes of the tables. In the experiments in which aminopterin was used, the injections of PGA and vitamin B_{12} were given one hour before the aminopterin. Corn oil was used as the injection vehicle for stilbestrol in all instances, and the appropriate control groups were given corn oil only, in the same dosage as was used for vehicle purposes. All injections were made subcutaneously. Vitamin B_{12} was given by injection in all experiments.

At autopsy the chicks were killed with chloroform and the oviducts immediately removed, blotted, and weighed on a Roller-Smith torsion balance.

RESULTS

Experiment 1M (Table 1): Supplementation of unfed, vitamin B_{12}-deficient pullets with vitamin B_{12} for three days, during which time high doses of stilbestrol were administered, did not produce a significantly greater response to the stilbestrol than occurred in those pullets not receiving supplementary vitamin B_{12}. A statistical comparison of the oviduct weights of Groups 2 and 4, however, did show a P value of 0.1–0.5, suggesting that under different experimental conditions a significantly greater response in the presence of supplementary vitamin B_{12} might be demonstrated.

In previous studies with normal White Leghorn pullets from a commercial hatchery, using experimental conditions parallel to those in Experiment 1M of this series, the mean body weight at autopsy was 33.0 ± 0.9 gm. and the mean oviduct weight in response to stilbestrol was 63.9 ± 3.5

[2] Product of the Glidden Company.

TABLE 1. (EXP. 1M): STILBESTROL ACTION IN STARVED, VITAMIN B_{12}-DEFICIENT PULLETS

Group No.	Injections		Autopsy data‡: Mean values ±S.E.§		
	B_{12}*	Stilb.†	No. of chicks	Body weight, gm.	Oviduct weight, mg.
1	0	0	5	29 ±0.8	6.9 ±0.7
2	0	+	9	29 ±0.7	40.6 ±2.9
3	+	0	5	29 ±1.5	8.3 ±0.9
4	+	+	11	29 ±0.8	49.6 ±3.6

* Vitamin B_{12}: 3 μg./0.1 ml. saline/day; days 1, 2 and 3.
† Diethylstilbestrol: 1 mg./0.1 ml. oil/day; days 1, 2 and 3.
‡ Autopsy on day 4, 24 hrs. after last injection.
§ Standard Error of Mean:

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

mg. This oviduct response was significantly greater than that observed in the vitamin B_{12}-deficient pullets (but not in the vitamin B_{12}-supplemented pullets) of Experiment 1M reported here, on the basis of either absolute oviduct weights or ratios of oviduct weight to body weight.

Experiment 2M (Table 2): All pullets were fed the basal ration and vitamin supplement from day one. On day six they were divided into experimental groups. Beginning at this time and continuing for five days, PGA was added to the vitamin supplement for all groups and injections of stilbestrol and vitamin B_{12} were given to the designated groups daily.

Under these conditions, oviduct growth in response to stilbestrol was significantly greater in the vitamin B_{12}-supplemented pullets than in the deficient pullets (Group 2 compared to Group 4). Stilbestrol inhibited body growth in the vitamin B_{12}-supplemented group but not in the deficient group. From these results it seemed possible that longer treatment with estrogen might serve to exaggerate further the difference in responsiveness to stilbestrol already shown to exist between vitamin B_{12}-deficient and vitamin B_{12}-supplemented pullets.

Experiments 13M and 15M (Table 3): In these experiments stilbestrol was administered for nine days. In addition, the PGA status of the pullets was tested.

TABLE 2. (EXP. 2M): STILBESTROL ACTION IN FED, VITAMIN B_{12}-DEFICIENT PULLETS

Group No.	Injections		Autopsy data‡: Mean values S.E.		
	B_{12}*	Stilb.†	No. of chicks	Body weight, gm.	Oviduct weight, mg.
1	0	0	5	53.4 ±4.7	13.8 ±1.4
2	0	+	8	51.1 +2.0	152.2 ±8.6
3	+	0	6	64.7 ±3.1	19.7 ±2.6
4	+	+	7	50.3 ±2.5	184.0 ±5.6

* Vitamin B_{12}: 3 μg./0.1 ml. saline/day; days 6–10.
† Diethylstilbestrol: 1 mg./0.1 ml. oil/day; days 8, 9 and 10.
‡ Autopsy on day 11, 24 hrs. after last injection.
All pullets received PGA, 4.5 mg. (0.3 ml. Folvite) per day by injection, days 6–10.

The six experimental groups possible in Experiment 13M permitted inclusion of controls not treated with stilbestrol. In Experiment 15M these controls were omitted, all groups having received stilbestrol, and a control group which was supplemented with PGA in the absence of additional vitamin B_{12} could then be included.

In both experiments supplementation with vitamin B_{12} permitted a 100% increase in oviduct weight in response to stilbestrol. Body growth was also significantly increased by the vitamin B_{12}-supplement, but not to the same extent as was the oviduct growth, so that on a ratio basis (mg. of oviduct per gram of body weight) oviduct weight was still significantly

TABLE 3. EFFECTS OF PROLONGED STILBESTROL ACTION IN VITAMIN B_{12}-DEFICIENT PULLETS

Exp. No.	Group No.	Treatment			Autopsy data§: Mean values ± S.E.			
		PGA*	B_{12}†	Stilb.‡	No. of chicks (Deaths)	Body wt., gm.	Oviduct wt., mg.	Oviduct (mg.) / Body (gm.)
13M	1	0	0	0	8 (1)	46.1 ± 1.3	13.6 ± 0.9	0.3 ± 0.02
	2	0	0	+	10 (2)	43.9 ± 0.5	527.9 ± 51.4	12.0 ± 1.00
	3	0	+	0	9 (0)	65.8 ± 3.0	24.1 ± 2.6	0.4 ± 0.05
	4	0	+	+	7 (2)	61.3 ± 1.8	1213.0 ± 44.6	19.0 ± 1.05
	5	+	+	0	7 (2)	67.1 ± 3.2	18.1 ± 0.9	0.3 ± 0.02
	6	+	+	+	8 (1)	75.4 ± 2.3	1421.3 ± 76.5	18.6 ± 0.70
15M	1	0	0	+	9 (2)	48.4 ± 2.3	602.6 ± 47.5	12.5 ± 0.9
	2	+	0	+	6 (4)	48.5 ± 2.2	820.8 ± 91.0	17.0 ± 1.6
	3	0	+	+	10 (0)	73.1 ± 2.6	1320.5 ± 50.4	18.0 ± 0.8
	4	+	+	+	10 (0)	75.3 ± 3.6	1431.8 ± 75.8	19.2 ± 0.7

* PGA: (in oral vitamin supplement) Exp. 13M: 0.1 mg. days 5–12; Exp. 15M: 0.2 mg., days 3–12.
† Vitamin B_{12}: Exp. 13M: 5 μg./0.1 ml. H_2O, days 6–12;
Exp. 15M: 6 μg./0.1 ml. H_2O, days 2–7; 10 μg./0.1 ml. H_2O, days 8–12.
† Stilbestrol: 1 mg./0.1 ml. corn oil/day, days 4–12 (total dose = 9 mg.).
§ Autopsy: At 13 days of age, 24 hours after last injection.

greater in the vitamin B_{12}-supplemented groups than in the vitamin B_{12}-deficient groups. The longer treatment with stilbestrol was, then, effective in producing greater disparity in response to stilbestrol between vitamin B_{12}-deficient and vitamin B_{12}-supplemented pullets.

In Experiment 13M the vitamin B_{12}-supplementation, although increased over that used in Experiment 2M, still seemed to be marginal, since the stilbestrol treatment again tended to limit body growth (Group 3 compared to Group 4). The PGA given in addition to the vitamin B_{12} completely overcame this tendency toward limited body growth, however, having permitted not only a significantly greater body weight but also simultaneously an increase in oviduct response which, on the basis of absolute oviduct weights (Group 4 compared to Group 6), approached statistical significance at the 5% level of confidence.

In Experiment 15M the daily allowance of vitamin B_{12} was increased still further and given for eleven days. Under these conditions, vitamin B_{12}

alone permitted the same effectiveness of the stilbestrol as seen in Experiment 13M, but without limitation of body weight. PGA given in addition to this dose of vitamin B_{12} did not result in significant increase in body weight nor in oviduct response to stilbestrol (Group 1 compared to Group 2; Group 3 compared to Group 4). A similar relationship of PGA to vitamin B_{12} dosage has previously been reported (Griffith and Nye, 1954).

Experiments 21M and 23M (Table 4): These experiments were attempts to obtain a PGA deficiency in vitamin B_{12}-deficient pullets, using aminopterin. All groups were given both aminopterin and stilbestrol. The experiments through Experiment 15M had been done with animals which had not been depleted of PGA and therefore could not furnish evidence with

TABLE 4. STILBESTEROL ACTION IN AMINOPTERIN-TREATED VITAMIN B_{12}-DEFICIENT PULLETS

Exp. No.	Group No.	Vitamins		Autopsy data‡: Mean values ±S.E.			
		PGA*	B_{12}†	No. of chicks (Deaths)	Body wt., gm.	Oviduct wt., mg.	Oviduct (mg.) / Body (gm.)
21M	1	+	0	7 (2)	51.7 ± 3.0	1080.0 ± 28.0	21.2 ± 0.9
	2	0	+	3 (7)	54.7 ± 3.8	836.0 ± 101.0	15.2 ± 0.8
	3	+	+	9 (1)	73.8 ± 2.5	1422.0 ± 48.0	19.6 ± 0.3
23M	1	0	0	4 (7)	45.5 ± 1.9	592.8 ± 84.0	12.9 ± 1.5
	2	+	0	6 (0)	56.0 ± 5.7	862.2 ± 134.0	15.2 ± 0.9
	3	0	+	8 (4)	53.3 ± 3.0	942.8 ± 68.4	17.6 ± 0.7
	4	+	+	6 (1)	57.5 ± 5.7	972.7 ± 66.4	17.2 ± 1.1

Treatments begun at 3 days of age unless otherwise stated.
All chicks received: (1 hr. after PGA and vitamin B_{12})
Aminopterin: Exp. 21M: 5 μg./0.1 ml. saline, daily except days 7 and 9.
Exp. 23M: 5 μg./0.1 ml. saline, days 3, 4 and 6; 2.5 μg. days 7, 9, 11 and 12.
Stilbestrol: 1 mg./day; Exp. 21M, total dose = 8 mg.; Exp. 23M, total dose = 11 mg.
* PGA: (injected) Exp. 21M, 4.5 mg./day; days 2–10; Exp. 23M, 3 mg./day, days 3–13.
† Vitamin B_{12}: 10 μg./0.1 ml. saline/day; Exp. 21M, days 2–10; Exp. 23M, days 3–13.
‡ Autopsy: 24 hrs. after last injection; Exp. 21M, at 11 days of age; Exp. 23M, at 14 days of age.

regard to the possible role of vitamin B_{12} in catalyzing the conversion of PGA to more active metabolites.

In Experiment 23M an amount of PGA (3 mg. per day) was administered which served, without permitting a significant increase in body growth or oviduct response to stilbestrol, to prevent the high mortality that occurred in the group given aminopterin alone (Group 1 compared to Group 2). In Group 3, vitamin B_{12} alone permitted a significant growth response in both body and oviduct. This growth response, however, was not significantly improved by the daily supplement of 3 mg. of PGA. In other words, an amount of PGA which just balanced the dose of aminopterin in the absence of supplementary vitamin B_{12}, was not increased in effectiveness by supplementary vitamin B_{12}.

In Experiment 21M, where a higher dose of PGA was used and where there appeared to be a small excess of PGA over the amount required just

to overcome the aminopterin given, the PGA alone, in vitamin B_{12}-deficient pullets was able to permit a significant increase in oviduct : body ratio over that which occurred in the aminopterin-treated pullets supplemented with vitamin B_{12} alone (Group 1 compared to Group 2). When vitamin B_{12} was given in addition to this dose of PGA, a significant additional increase occurred in both oviduct and body weights, although the oviduct:body weight ratio remained unchanged from that which occurred when the PGA was given alone (Group 1 compared to Group 3). The effectiveness of the vitamin B_{12}, then, was limited by an aminopterin-induced deficiency of PGA; and conversely, the effectiveness of PGA was limited by a deficiency

TABLE 5. STATISTICAL ANALYSIS OF DATA

Exp. No.	Groups compared	Basis of comparison					
		Body weight		Oviduct weight		$\frac{\text{Oviduct}}{\text{Body}}$ ratio	
		t*	P	t*	P	t*	P
1M	2 and 4	—	—	1.96	0.1–0.05	—	—
2M	1 and 3	2.00	0.1–0.05	—	—	—	—
	3 and 4	2.72	0.02–0.01	—	—	—	—
	2 and 4	—	—	3.14	<0.01	—	—
13M	2 and 4	—	—	—	—	5.27	0.001
	4 and 6	4.80	<0.001	2.34	0.05–0.02	—	—
15M	1 and 2	—	—	2.09	0.1–0.05	2.40	0.05–0.02
21M	1 and 2	—	—	2.37	0.05–0.02	1.66	0.2
	1 and 3	—	—	6.18	<0.001	1.30	0.3
	2 and 3	—	—	—	—	5.41	<0.001
23M	1 and 2	1.75	0.2	0.38	>0.5	1.32	0.3
	1 and 3	2.20	0.05	3.38	<0.01	2.19	0.05
	2 and 4	—	—	0.74	0.5	1.25	0.3

* Fisher's t value.

of vitamin B_{12}. Under the conditions used, no increase in effectiveness of PGA in permitting oviduct response to stilbestrol was brought about by vitamin B_{12}. These results are in agreement with other types of evidence (Welch and Nichol, 1952; Jukes and Williams, 1954) which have indicated that vitamin B_{12} does not function to promote the conversion of PGA to active metabolites.

DISCUSSION

It is impossible to formulate more than a hypothetical, partial interpretation of the experimental data in terms of metabolic reactions since, among other reasons, the biochemical functions and relationships of PGA and vitamin B_{12} are not entirely elucidated. Because these vitamins have been implicated in the metabolism of one carbon units, it is tempting to speculate, especially since the diet was low in methionine, that this amino

acid is one of the metabolites which became limiting in oviduct response to stilbestrol in vitamin B_{12}-deficient pullets (in Experiments 1M through 21M), and to predict that addition of homocystine to the diet would have permitted even greater body growth and response to stilbestrol in the vitamin B_{12}-supplemented pullets. Under the conditions used in these experiments, one of the functions of vitamin B_{12} might have been to permit more efficient conservation (or re-utilization) of the metabolically available homocysteine through promoting better synthesis of methyl groups. Alternatively or simultaneously, low purine biosynthesis may have been limiting tissue proliferation, since one carbon units are essential to purine formation.

Because inanition controls were not included the possibility that the effects of vitamin B_{12}-supplementation can be explained on the basis of increased dietary intake has not been entirely ruled out.

Since, of all the vitamin deficiencies investigated, deficiences of vitamin B_{12} and PGA are the only ones in which stilbestrol stimulation of the oviduct is significantly reduced, and since both of these vitamins have been implicated in the intermediary metabolism of one carbon units, it is attractive to postulate that the impairment in oviduct response in both deficiences occurs within the same group of enzymatic reactions. Other possibilities also exist, however, such as each vitamin being essential to the biosynthesis of different compounds necessary to tissue proliferation.

SUMMARY

In vitamin B_{12}-deficient pullets, maintained on a diet deficient in vitamin B_{12} and low in choline and methionine, oviduct response to stilbestrol was significantly lower than in controls resupplemented with vitamin B_{12}. It is therefore concluded that vitamin B_{12} is essential to optimal response of the chick oviduct to stilbestrol.

Supplements of vitamin B_{12} were unable to increase the effectiveness of pteroylglutamic acid (PGA) in overcoming aminopterin inhibition of stilbestrol action in vitamin B_{12}-deficient pullets receiving enough PGA just to overcome the aminopterin administered. These results are consistent with the idea that vitamin B_{12} is related to the action of PGA otherwise than as a catalyst which promotes the conversion of PGA to metabolically active forms.

Acknowledgments

The author wishes to thank Drs. R. I. Dorfman, G. R. Greenberg, L. C. Norris, J. A. Muntz, W. Sakami and M. F. Utter for critical reading of the manuscript. Valuable technical assistance was given by Donald Nemec, William Beard and Jerome Dudley.

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NOTES AND COMMENTS

EFFECT OF THYROXINE ON THE PHOSPHATASE ACTIVITY OF PIGEON SKIN

It has been postulated by Jeener (1948) and Bradfield (1950) that alkaline phosphatase activity is associated with the intracellular synthesis of fibrous protein. On the other hand, it is well known that, in the birds, the administration of thyroid substance induces molting of feathers and cornification of epidermis. Formation of new feathers and cornification of epidermis must be accompanied by the synthesis of fibrous protein. The present investigation was undertaken to study the relation between the keratinization or cornification caused by thyroxine in the skin of pigeons and the activity of acid and alkaline phosphatases of the tissue.

EXPERIMENTAL

Carrier pigeons of either sex weighing between 300 and 500 gm. received a single intramuscular injection of 10 mg. of thyroxine (Roche) dissolved in 1.5 ml. of slightly alkaline solution of sodium hydroxide. The birds injected with the same amount of sodium hydroxide solution served as controls. All the pigeons were sacrificed four days after the injection. Phosphatase activity was determined on the non-feathered and feathered areas of ventral skin. The feathers were plucked away and then the skin was removed. The skin dissected out was weighed and cut into small bits with scissors after adding a drop of distilled water. The brei was transferred drop by drop into a glass-rod homogenizer and ground as finely as possible. The assay of tissue phosphatase activity was performed, using the standard system consisting of 1.0 ml. of 0.15 M histidine buffer, 1.0 ml. of 1% sodium glycerophosphate, 0.2 ml. of 0.1 M $MgCl_2$ as an activator, 0.5 ml. of tissue homogenate and 0.3 ml. of water to a total volume of 3.0 ml. The enzymatic reaction was started by addition of the homogenate, equivalent to 30 mg. of wet tissue, to the reaction mixture. The reaction was stopped by adding 1.0 ml. of 25% trichloracetic acid after an incubation of 3 hours at 37° C. with occasional shaking. Acid phosphatase activity was measured at pH 5.6 and alkaline one at pH 9.2, adjusted by addition of KOH. The control runs without substrate and without homogenate were always paralleled. The deproteinized filtrate was assayed for inorganic phosphate by the method of Lohmann and Jendrássik (1926), using an electrophotometer. The phosphatase activity is given as μg. inorganic phosphate (P) liberated in 3 hours at 37° C. per 30 mg. of wet tissue.

For the study of distribution of alkaline and acid phophatases in the skin, histochemical tests were made essentially according to the method of Gomori on a piece of the ventral skin, either feathered or non-feathered, of the pigeons used for the determination of the enzymes. The tissue was fixed in cold acetone for 24 hours. After dehydrating, the tissue was imbedded in paraffin and sections were cut 6μ in thickness. For the demonstration of acid phosphatase, the following somewhat modified reaction mixture was employed: 12 ml. of $\frac{1}{3}$ M acetate buffer of pH 4.6, 10 ml. of 1/10 M lead nitrate, 78 ml. of H_2O and 0.5 gm. of sodium glycerophosphate.

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RESULTS

Determination of alkaline and acid phosphatases of the skin. The results are summarized in Table 1. In alkaline phosphatase activity there was no significant difference between the feathered and non-feathered areas of the ventral skin of intact birds. Following the injection of thyroxine the enzyme activity increased significantly in both

TABLE 1. PHOSPHATASE ACTIVITY OF VENTRAL SKIN OF PIGEON AT 4 DAYS AFTER A SINGLE INJECTION OF 10 MG. THYROXINE. ACTIVITY IS PRESENTED AS μG. P LIBERATED IN 3 HOURS AT 37° C. AT pH 5.6 (ACID) OR AT pH 9.2 (ALKALINE) PER 30 MG. WET TISSUE IN THE PRESENCE OF 6.6 MM $MgCl_2$

Sample	Phosphatase activity			
	Feathered area		Non-feathered area	
	acid	alkaline	acid	alkaline
Thyroxine-injected	109.5	5.9	109.5	2.9
	98.6	6.5	87.3	3.7
	77.0	5.9	73.1	4.5
	(95.0)*	(6.1)	(90.0)	(3.7)
Control	44.9	2.5	58.0	1.9
	42.2	5.0	35.0	0.3
	35.0	2.7	45.0	1.7
	(40.7)	(3.4)	(46.0)	(1.3)

* Numbers in parentheses are averages.

areas ($p<0.05$) and it was significantly higher in the feathered area than in the non-feathered one ($p<0.01$).

Acid phosphatase activity of the ventral skin was much greater than that of alkaline phosphatase. No significant difference was found in the activity between the feathered and non-feathered areas. Thyroxine injection produced about a two-fold increase in this enzyme concentration equally in both areas.

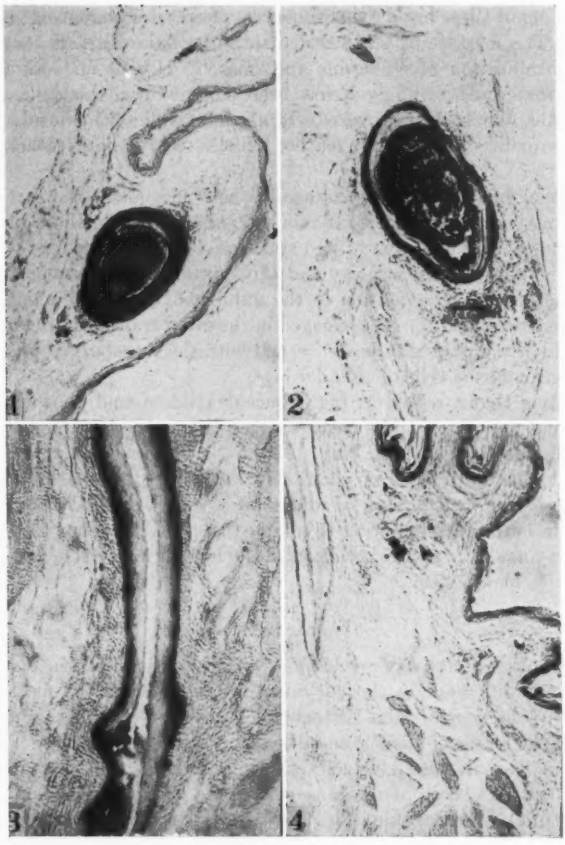
In order to compare the activity of skin acid phosphatase with that of other tissues, liver, kidney and spleen of pigeons were used. Although the enzyme activity of these organs was several times higher than that of the skin, the activating effect of thyroxine on acid phosphatase was much less in these organs than in the skin (Table 2).

Distribution of alkaline and acid phosphatases of the skin. Deposits of cobalt sulfide, indicating alkaline phosphatase activity, were dense in the cells of cylinder layer, the lower part of epidermis of the feather follicle, and the pulp of feather papilla (Figs. 1, 2 and 3).

TABLE 2. ACID PHOSPHATASE ACTIVITY OF SEVERAL TISSUES OF PIGEON AT 4 DAYS AFTER A SINGLE INJECTION OF 10 MG. THYROXINE. ACTIVITY IS GIVEN AS IN TABLE 1

Tissue	Phosphatase activity	
	Thyroxine-injected	Control
Skin*	77	35
Liver	324	254
Kidney	295	226
Spleen	254	204

* Feathered area of ventral skin.



FIGS. 1 and 2. Section through ventral skin of normal pigeon showing alkaline phosphatase in pulp and cylinder layer of feather follicle. ×380.

FIG. 3. Section through ventral skin of normal pigeon showing alkaline phosphatase in epidermis of feather follicle. ×120.

FIG. 4. Section through ventral skin of normal pigeon showing acid phosphatase in epidermal layer. Note epidermis of feather follicle without acid phosphatase. ×100.

On the other hand, acid phosphatase activity was demonstrated strongly in the epidermal layer of the skin. This phosphatase was absent in the epidermis of the feather follicle (Fig. 4). Quantitative difference in the activity of these enzymes between the skin of experimental and control birds was not evident histochemically under the present experimental conditions.

DISCUSSION

The increase of alkaline phosphatase activity of the non-feathered area of the ventral skin of thyroxine-birds may be associated with a marked cornification of epidermis. At

the time of killing of these birds the cornified layer was desquamating severely in pieces from the skin. The increase of this enzyme activity of the feathered area may be concerned with cornification of epidermis and, moreover, with an activation of feather follicle by thyroxine. Accordingly it has been demonstrated histochemically that the tissues of feather follicle contain relatively abundant alkaline phosphatase (Figs. 1, 2 and 3), and thyroxine stimulates the feather follicles to form new feathers (cf. Takewaki and Mori, 1944).

The occurrence of alkaline phosphatase in the epidermis of the feather follicle and cylinder layer may have an important role in keratinization in these tissues. The findings support the concept of Jeener (1948) and Bradfield (1951) dealing with the relation between alkaline phosphatase activity and the synthesis of fibrous protein.

Moog (1946) has drawn attention to the number of organs in which alkaline phosphatase has been located in the cells engaged in the rapid transport of materials. Possibly the phosphatase of the pulp may be concerned with the transport of organic substances between the pulp and the cells of cylinder layer.

It is interesting that a relatively high concentration of acid phosphatase was found in the skin of pigeons, since it has been reported that in mammalian skin this enzyme activity is negligible (cf. Folly and Kay, 1936).

Acid phosphatase activity was about the same both in the feathered and non-feathered areas of the ventral skin of intact birds and it showed about a two-fold increase equally in both areas following thyroxine injection. It seems, therefore, that acid phosphatase may not have a direct relation to the feather formation, but may be related to general cellular metabolism.

SUMMARY

Alkaline phosphatase activity was found very weak in the skin of pigeons. There was no significant difference in the enzyme activity between the feathered and non-feathered areas of the ventral skin. Histochemical reaction for this enzyme was stronger in the tissues of the feather follicle than in the other tissues of the skin. Thyroxine injection caused an increase in this enzyme activity in both areas.

High concentration of acid phosphatase was found equally in the feathered and non-feathered areas of the ventral skin. This enzyme was localized chiefly in the epidermal layer of the skin. A marked increase in acid phosphatase activity appeared after injection of thyroxine in both areas of the ventral skin. This thyroxine effect on acid phosphatase was much more evident in the skin than in the liver, spleen and kidney.

The implications of these findings are discussed in relation to the morphological changes caused by thyroxine in the skin of pigeons.

H. Kobayashi, K. Maruyama and S. Kambara

Zoological Institute
Faculty of Science
Tokyo University
Tokyo, Japan

Acknowledgment

It is a pleasure to thank Professors K. Takewaki and J. Ishida for their kind interest.

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THE SEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE INDIANA UNIVERSITY SCHOOL OF MEDICINE AT THE CONTINUATION STUDY CENTER OF THE INDIANA UNIVERSITY MEDICAL CENTER, INDIANAPOLIS, INDIANA— SEPTEMBER 26–OCTOBER 1, 1955

Monday (AM) September 26, 1955

8:00 to 8:45 Registration: Place—*Lobby—Indiana State Board of Health Building.*
Introductions—Dr. John D. Van Nuys, Dean, Indiana University School of Medicine.
Dr. Don E. Wood, Chairman of Local Committee on Arrangements.
Dr. Roberto F. Escamilla, Chairman of Postgraduate Committee of Endocrine Society.
Chairman of morning session—Dr. Don E. Wood.

9:00 to 9:45 Anterior pituitary, anatomy and present status of physiology—relation to hypothalamus—Dr. Charles W. Lloyd.

9:45 to 10:30 Hypopituitarism—Dwarfism, Simmonds' Disease, Sheehan's Syndrome, Froelich's Syndrome—Dr. Roberto F. Escamilla.

10:30 to 10:45 Intermission.

10:45 to 11:30 Hyperpituitarism—Gigantism and Acromegaly—Dr. Karl E. Paschkis.

11:30 to 12:00 Posterior pituitary and intermediate lobe anatomy and physiology—relation to hypothalamus—Dr. Charles W. Lloyd.

12:00 to 12:30 Diabetes Insipidus—role of posterior pituitary in fluid balance and hypertension—Dr. Charles W. Lloyd.

12:30 to 2:00 Lunch.

Monday (PM)

Chairman of afternoon session—Dr. Karl E. Paschkis.

2:00 to 2:30 Examination of the patient for endocrine disease—Dr. Frank L. Engel.

2:30 to 3:00 Place of the laboratory in endocrine diagnosis—Dr. Roberto F. Escamilla.

3:00 to 3:15 Intermission.

3:15 to 4:30 Demonstration of laboratory procedures—Dr. Robert B. Greenblatt and Dr. Don E. Wood.

4:30 to 5:00 Question and answer period.

6:30 Get-acquainted party—Cocktails and dinner for faculty and students, sponsored by Eli Lilly & Company, Indianapolis Athletic Club.

Tuesday (AM) September 27, 1955

Chairman of morning session—Dr. Frank L. Engel.

9:00 to 9:30 Thyroid, anatomy and physiology—relation to pituitary—Dr. Brown M. Dobyns.

9:30 to 10:00 Goiter—classification and present concepts—Dr. Rulon W. Rawson.

10:00 to 10:40 Hypothyroidism—in childhood and in adults—Dr. Henry H. Turner.

10:40 to 10:50 Intermission.

10:50 to 11:30 Hyperthyroidism—Dr. Rulon W. Rawson.
11:30 to 12:00 Thyroid cancer—Dr. Brown M. Dobyns.
12:00 to 12:30 Thyroiditis—Dr. Rulon W. Rawson.
12:30 to 2:00 Lunch.

Tuesday (PM)

Chairman of afternoon session—Dr. Rulon W. Rawson.

2:00 to 2:30 Parathyroids—anatomy and physiology—Dr. Edward C. Reifenstein, Jr.
2:30 to 3:15 Hypoparathyroidism—Dr. Glenn W. Irwin.
3:15 to 3:30 Intermission.
3:30 to 4:15 Hyperparathyroidism—Dr. Frank L. Engel.
4:15 to 4:45 Metabolic bone disease—differential diagnosis and treatment—Dr. Edward C. Reifenstein, Jr.
4:45 to 5:30 Question and answer period.

Wednesday (AM) September 28, 1955

Chairman of morning session—Dr. Lawson Wilkins.

9:00 to 9:45 Adrenal Cortex—anatomy, chemistry and physiology—Dr. Ralph I. Dorfman.
9:45 to 10:30 Adrenal Insufficiency—Dr. Frank L. Engel.
10:30 to 10:45 Intermission.
10:45 to 11:30 Cushing's Disease and Cushing's Syndrome—Dr. Robert B. Greenblatt.
11:30 to 12:00 Adrenogenital syndrome, including congenital type—diagnosis and treatment—Dr. Lawson Wilkins.
12:00 to 12:30 Other adrenal cortical tumors; Aldosteronism—Dr. Karl E. Paschkis.
12:30 to 2:00 Lunch.

Wednesday (PM)

Chairman of afternoon session—Dr. Ralph I. Dorfman.

2:00 to 2:45 Hermaphroditism: Classification, Diagnosis, Selection of Sex of Rearing, Treatment—feminizing adrenal tumors—Dr. Lawson Wilkins.
2:45 to 3:30 Use of cortisone, corticotropin and related substances—Management of patient before and after adrenal surgery—Dr. Frank L. Engel.
3:30 to 3:45 Intermission.
3:45 to 4:30 Hyperinsulinism and Spontaneous Hypoglycemia—Dr. Edward H. Rynearson.
4:30 to 5:00 Obesity and Leanness—Dr. Edward H. Rynearson.
5:00 to 5:30 Question and answer period.

Thursday (AM) September 29, 1955

Chairman of morning session—Dr. Edward C. Reifenstein, Jr.

9:00 to 9:30 The testis—anatomy and physiology—Dr. Earl T. Engle.
9:30 to 10:15 Male Hypogonadism—Dr. Henry H. Turner.
10:15 to 10:30 Intermission.
10:30 to 11:00 Cryptorchidism—Dr. Robert Garrett.
11:00 to 11:30 Male climacteric—Impotence—Dr. Henry H. Turner.
11:30 to 12:15 Testicular tumors—including male pseudohermaphroditism—Dr. Robert Garrett.
12:15 to 2:00 Lunch.

Thursday (PM)

Chairman of afternoon session—Dr. Willard M. Allen.

2:00 to 2:30 Hormones and cancer—present knowledge—Dr. Ralph I. Dorfman.
2:30 to 3:00 Male infertility—present status of therapy—Dr. Karl E. Paschkis.
3:00 to 3:30 Gynecomastia—Klinefelter's syndrome—Dr. Don E. Wood.
3:30 to 3:45 Intermission.
3:45 to 5:00 Case presentations—pituitary, thyroid, parathyroids, adrenals, testes—Dr. Don E. Wood; *and resident physicians, Indiana University Medical Center.*
5:00 to 5:30 Questions and answers.

Friday (AM) September 30, 1955

Chairman of morning session—Dr. Robert B. Greenblatt.

9:00 to 9:45 The ovary—anatomy and physiology—Dr. Earl T. Engle.
9:45 to 10:45 Ovarian hypofunction syndromes—including female eunuchoidism, ovarian aplasia, Turner's syndrome—Dr. Henry H. Turner.
10:45 to 11:00 Intermission.
11:00 to 11:30 Ovarian tumors of endocrine significance—Dr. Carl P. Huber.
11:30 to 12:30 Functional ovarian disorders, including meno- and metrorrhagia—Dr. Willard M. Allen.
12:30 to 2:00 Lunch.

Friday (PM)

Chairman of afternoon session—Dr. Earl T. Engle.

2:00 to 2:30 The menopause—Dr. Willard M. Allen.
2:30 to 3:00 Female infertility—Dr. Robert B. Greenblatt.
3:00 to 3:15 Intermission.
3:15 to 4:30 Endocrine problems of pregnancy—Dr. Sprague H. Gardiner.
4:30 to 5:30 Case presentations—ovaries. Questions and answers. Dr. Don E. Wood; *and resident physicians, Indiana University Medical Center.*
6:30 Banquet of the Course, including students and faculty. Cost *$4.00.* Tickets will be sold at time of registration. *Room M-124, Student Union Bldg.*, Indiana University Medical Center.
Speaker: Dr. Carl P. Huber—"Stress during labor."

Saturday (AM) October 1, 1955

Chairman of the morning session—Dr. Roberto F. Escamilla.

9:00 to 9:30 Simple Hirsutism—Stein-Leventhal syndrome—Dr. Willard M. Allen.
9:30 to 10:15 Adrenal medulla—Pheochromocytoma—Dr. Glenn W. Irwin.
10:15 to 10:30 Intermission.
10:30 to 10:45 Chemistry and actions of available insulins—Dr. Franklin B. Peck, Sr.
10:45 to 12:00 Essentials in management of uncomplicated diabetes—Dr. Charles E. Test.
12:00 to 12:30 Pointers in management of complications of diabetes—Dr. Franklin B. Peck, Sr.

For further information concerning the program, write Post Graduate Office, Indiana University Medical Center, 1100 West Michigan Street, Indianapolis 7, Indiana. Registration is limited to 100; tuition fee is $100.00. Arrangements for housing accommodations will be made through the Indiana Medical Center.

FACULTY

Willard M. Allen, M.D., Professor of Obstetrics & Gynecology, Washington University School of Medicine, St. Louis, Missouri.

Brown M. Dobyns, M.D., Associate Professor of Surgery, Western Reserve School of Medicine, Cleveland, Ohio.

Ralph I. Dorfman, Ph.D., Associate Director, Worcester Foundation, and Research Professor of Biochemistry, Boston University School of Medicine, Shrewsbury, Mass.

Frank L. Engel, M.D., Associate Professor of Medicine, Assistant Professor of Physiology, Duke University, Durham, North Carolina.

Earl T. Engle, Ph.D., Professor of Obstetrics & Gynecology, Columbia University, College of Physicians and Surgeons, New York City, New York.

Roberto F. Escamilla, M.D., Associate Clinical Professor of Medicine, University of California School of Medicine, San Francisco, California.

Sprague H. Gardiner, M.D., Associate Professor of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, Indiana.

Robert A. Garrett, M.D., Professor of Urology, Indiana University School of Medicine, Indianapolis, Indiana.

Robert B. Greenblatt, M.D., Professor of Endocrinology, Medical College of Georgia, Augusta, Georgia.

Carl P. Huber, M.D., Chairman, Department of Obstetrics and Gynecology Indiana University School of Medicine, Indianapolis, Indiana.

Glenn W. Irwin, M.D., Assistant Professor of Medicine, Indiana University School of Medicine, Indianapolis, Indiana.

Charles W. Lloyd, M.D., Assistant Professor of Obstetrics and Assistant Professor of Medicine, State University of New York, Syracuse, New York.

Karl E. Paschkis, M.D., Associate Professor of Medicine; Associate Professor of Physiology; Director, Division of Endocrine and Cancer Research, The Jefferson Medical College of Philadelphia, Philadelphia, Pennsylvania.

Franklin B. Peck, Sr., M.D., Associate Professor of Medicine, Indiana University School of Medicine; Director, Medical Research Cooperation, Lilly Research Laboratories, Indianapolis, Indiana.

Rulon W. Rawson, M.D., Professor of Medicine, Sloan-Kettering Division of Cornell University Medical School, New York City, New York.

Edward C. Reifenstein, Jr., M.D., Associate Medical Director, E. R. Squibb & Sons, Division of Olin Mathieson Chemical Corporation; Assistant Clinical Professor of Medicine, New York Medical College, New York City, New York.

Edward H. Rynearson, M.D., Chairman of Sections, Mayo Clinic, Rochester, Minn.

Charles E. Test, M.D., Assistant Professor of Medicine, Indiana University School of Medicine, Indianapolis, Indiana.

Henry H. Turner, M.D., Clinical Professor of Medicine, University of Oklahoma School of Medicine; Chief, Endocrine Clinic, University Hospitals, Oklahoma City, Oklahoma.

Lawson Wilkins, M.D., Associate Professor of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Don E. Wood, M.D., Associate Professor, Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana.

INTER-SOCIETY CYTOLOGY COUNCIL

Inter-Society Cytology Council, third annual meeting, Statler Hotel, Cleveland, Ohio, Friday and Saturday, November 11 and 12, 1955. Everyone interested in Cytology is invited to attend.

Morning and afternoon sessions will comprise sixteen formal papers and nine round table discussions of cytological problems. Also, a symposium on "Problems of Early Endometrial Cancer."

Annual banquet, Friday evening, November 11. Annual business meeting, November 12 at 12:30 P.M.

Additional information may be obtained from Office of Secretary-Treasurer, 634 North Grand Avenue, St. Louis 3, Missouri.

AMERICAN CANCER SOCIETY

Applications for Grants in Cancer Research

Acting for the American Cancer Society, the Committee on Growth of the National Academy of Sciences-Research Council is accepting applications for grants-in-aid for cancer research in the United States. Applications received before October 1 will be considered during the winter and grants recommended at that time become effective on July 1, 1956. *Investigators now receiving support will be notified regarding application for renewal.*

The Committee feels that an understanding of cancer depends upon a deeper insight into the nature of the growth process, normal and malignant. Therefore, the scope of the research program is broad and includes, in addition to clinical investigations on cancer, fundamental studies in the fields of cellular physiology, morphogenesis, genetics, virology, biochemistry, metabolism, nutrition, cytochemistry, physics, radiobiology, chemotherapy, endocrinology and carcinogenesis. The Committee is particularly interested in encouraging research in the epidemiology of cancer.

Application blanks may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D. C.